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(54) Title: ANTIBODIES FOR MODULATING CD47-MEDIATED NEUTROPHIL TRANSMIGRATION

(57) Abstract

The present invention relates to methods and compositions for modulating CD47-mediated PMN transmigration across a cell layer. Monoclonal antibodies and functionally active antibody fragments which specifically bind to the CD47 antigen are provided. These antibodies and fragments are useful in screening assays to identify pharmaceutical lead compounds which likewise are capable of modulating CD47-mediated PMN transmigration across a cell layer. Methods and pharmaceutical compositions for modifying the immune response of a subject also are provided. The antibodies and fragments are based upon, or derived from, the C5/D5 antibody having ATCC Accession No. HB-12021.

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ANTIBODIES FOR MODULATING CD47-MEDIATED NEUTROPHIL TRANSMIGRATION

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Field of the Invention

This invention relates generally to the field of immunology and specifically to monoclonal antibodies which bind to CD47.

Background of the Invention

Active inflammation of surfaces lined by columnar epithelia is histologically defined by transmigration of neutrophils (PMN) across such epithelial monolayers and subsequent collection of PMN in the lumen (Kumar, N.B. et al. (1982) Am. J. Surg. Path. 6:523-9; Yardley, J.H. (1986), Recent Developments in the Therapy of Inflammatory Bowel Disease, J.H. Yardley, editor, Johns Hopkins, Baltimore MD, 3-9). Recently, neutrophils have been recognized not only to influence epithelial function during transmigration, but also to interact with biochemically distinct apical domains after translocation to the lumenal compartment, thus further modifying key epithelial processes (Madara, J.L. et al. (1993) J. Clin. Invest. 91:2320-2325; Strohmeier, G.R. et al. (1995) J. Biol. Chem. 270:2387-2394). For example, in intestinal epithelia it appears that PMN transepithelial migration may reversibly influence epithelial barrier function (Evans, C.W. et al. (1983) Br. J. Exp. Path. 64:644-54; Nash, S. et al. (1987) J. Clin. Invest. 80:1104-13; Parsons, P.E. et al. (1987) Am. J. Pathol. 129:302-12). Further, the arrival of PMN in the lumenal space reportedly results in interactions promoting electrogenic Cl^- secretion (Madara, J.L. et al. (1992) J. Clin. Invest. 89:1938-44; Madara, J.L. et al. (1993) J. Clin. Invest. 91:2320-2325), the known basis for secretory diarrhea (Donowitz, M. and M.J. Welsh (1987), Physiology of the gastrointestinal tract, Vol. 2., L.R. Johnson, editor,. Raven Press, New York,. 1351-1388). Thus, specific events related to the transmigration process may culminate the barrier and transport alterations characteristic of the epithelial dysfunction present in acutely inflamed mucosal surfaces.

The process by which PMN migrate across polarized columnar epithelial surfaces is only partially understood. It is increasingly clear that the paradigm which applies to PMN-endothelial interactions does not apply to PMN - epithelial interactions (Parkos, C.A. et al. (1994) J. Amer. Soc. Nephrol. 5:138-152). For example, PMN interactions with epithelia and endothelia display

contrasting dependencies on PMN β_2 integrins (Lo, S.K. et al. (1989) J. Immunol. 143:3325-29; Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479; Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12; Smith, C.W. et al. (1989) J. Clin. Invest. 83:2008-17) and carbohydrates (Colgan, S.P. et al. (1995) J. Biol. Chem. 270:10531-10539). Such interactions are also differentially regulated by inflammatory cytokines (Colgan, S.P. et al. (1993) J. Cell. Biol. 120:785-798; Colgan, S.P. et al. (1994) J. Immunol. 153:2122-2129). Likewise, ligands crucial to transendothelial movement of PMN such as the immunoglobulin superfamily members ICAM-1 and ICAM-2, reportedly are not expressed in columnar epithelia (ICAM-2 (de Fourgerolles, A. et al. (1991) J. Exp. Med. 174:253-67)). Such contrasting paradigms for PMN association with and transmigration across endothelia versus columnar epithelia are perhaps to be expected given the markedly different microenvironments and geometries of these highly divergent systems. However, absent a complete understanding of the identity and nature of the ligands responsible for PMN transmigration, little, if any, progress can be made in the development of therapeutic agents for modulating the interactions of PMN with any cell layer in vivo.

Summary of the Invention

Compositions and methods for modulating the transmigration of PMN across a cell layer (e.g., an epithelial cell layer, an endothelial cell layer) or through an extracellular matrix are disclosed herein. Although not intending to be bound by any particular theory, our observations that CD47 is expressed on colonic epithelium, as well as on PMN, have suggested to us that CD47 may be utilized by both epithelia and PMN during transmigration of PMN across columnar epithelia in vivo. Thus, in its broadest aspects, the invention relates to agents which modulate CD47-mediated transmigration of PMN or other CD47-expressing cells across cell layers and/or through an extracellular matrix. In particular, the invention relates to agents which modulate such transmigration across epithelial cell layers. Processes involving the use of such agents and methods of preparing and selecting agents having the requisite properties for modulating CD47-mediated PMN transmigration also are disclosed herein.

According to one aspect of the invention, inhibitory agents that inhibit neutrophil transmigration across a cell layer or through an extracellular matrix are provided. Two categories of such inhibitory agents are embraced within the instant invention: (1) antibodies or functionally-active antibody fragments that are related to, or derived from, the monoclonal antibody having ATCC Accession No. HB-12021 (also referred to herein as the "C5/D5"

antibody) deposited at the American Type Culture Collection (ATCC), Rockville, MD, on January 17, 1996 and (2) "epitopic" peptides that are related to, or derived from, the epitope on CD47 to which the deposited C5/D5 antibody specifically binds (referred to herein as the "CD47 epitope").

In a particularly preferred embodiment, the inhibitory agent is the C5/D5 antibody (ATCC Accession No. HB-12021). As will be immediately apparent to one of ordinary skill in the art, functionally active fragments of the C5/D5 monoclonal antibody which bind to the CD47 epitope also can be used to modulate CD47-mediated PMN transmigration. Functionally active fragments include the following: $F(ab')_2$ fragments, Fab' fragments, Fv fragments and Fd fragments. As used throughout this description, the term "antibodies" in reference to the invention is meant to embrace intact functionally-active antibodies as well as functionally-active fragments thereof.

In a related aspect of the invention, monoclonal antibodies having the characteristics of the C5/D5 antibody are provided. Such characteristics include structural characteristics (e.g., epitope specificity, paratope sequence), as well as functional characteristics (e.g., the inhibitory concentration of an antibody in a transmigration assay such as the in vitro transmigration screening assay disclosed in the Examples). The antibody having the characteristics of the C5/D5 antibody specifically recognizes the CD47 epitope, i.e., that portion of CD47 that is specifically recognized by the monoclonal antibody having ATCC Accession No. HB-12021. The CD47 epitope is defined, at least in part, by one or more amino acid sequences located within SEQ. I.D. No. 1. It is believed that the CD47 epitope is more particularly defined by an amino acid sequence located within a portion of SEQ. I.D. No. 1, identified herein as SEQ. I.D. No. 2. The amino acid sequence(s) which define the CD47 epitope contain between three and twenty amino acids, more preferably between four and twelve amino acids within SEQ. I.D. Nos. 1 and/or 2. The epitope is more particularly defined by one or more amino acid sequences selected from the group consisting of SEQ. I.D. Nos. 3-35. Preferably, the CD47 epitope is defined by a sequence selected from the group consisting of SEQ. I.D. Nos. 10-23 and 31-33, more preferably by SEQ. I.D. Nos. 17-23 and 31-33, and most preferably by SEQ. I.D. No. 17 or SEQ. I.D. No. 31.

The antibody having the "characteristics" of the C5/D5 antibody has a paratope (i.e., antigen-binding region) which is substantially identical to the paratope of the deposited C5/D5 antibody. The amino acid sequence of the C5/D5 paratope can be determined using no more

than ordinary skill in the art using conventional microsequencing techniques such as those referenced in the Examples. The preferred antibodies of the invention are characterized in having a paratope which has an amino acid sequence that is identical to the amino acid sequence of the C5/D5 antibody paratope. Amino acid sequence analysis of the C5/D5 antibody paratope allows the design and synthesis of novel antibodies and related functionally active fragments which specifically bind to the CD47 epitope and which exhibit substantially the same inhibitory concentration as the C5/D5 antibody in a transmigration assay.

The antibody having the "characteristics" of the C5/D5 antibody has an inhibitory concentration in a cell transmigration assay that is substantially identical to the inhibitory concentration of the C5/D5 antibody in the same type of assay. The transmigration assays of the invention measure the transmigration of neutrophils, as well as the migration of other CD47-expressing cells, across a support selected from the group consisting of a cell layer, an extracellular matrix layer (e.g., a layer containing exemplary extracellular matrix proteins and proteoglycans) and a cellular filter (e.g., a Boyden chamber). In view of a possible role for CD47 in modulating the function of $\alpha_v \beta_3$, an integrin implicated in for example, angiogenesis and tumor metastasis, we believe that agents which modulate CD47-mediated migration (the antibodies and epitopic peptides of the invention) should also be useful for modulating angiogenesis and tumor metastasis by affecting $\alpha_v \beta_3$ function.

Preferably, the migration assays of the invention are used to measure the transmigration of neutrophils across a cell layer (e.g., an epithelial cell layer, an endothelial cell layer) or through an extracellular matrix. In the particularly preferred embodiments, the assay is used to measure the transmigration of neutrophils across a polarized cell layer (e.g, in an apical-to-basolateral or basolateral-to-apical direction). The preferred antibodies of the invention inhibit neutrophil transmigration in a bidirectional fashion and/or do not inhibit CD11b/CD18-mediated neutrophil adhesion to the cells of the cell layer.

In the preferred embodiments, the antibodies having the characteristics of the C5/D5 antibody, have inhibitory concentrations in the transmigration assay that result in a least 65 to 75% inhibition of neutrophil migration. Preferably, the antibody has an inhibitory concentration that results in at least 80%, more preferably 85% and most preferably 90% inhibition of neutrophil transmigration in the assay. In general, the inhibitory concentrations of the antibodies in these assays fall between about 0.1μg/ml and 50μg/ml, inclusive. However, in the preferred embodiments, the antibodies are more potent and exhibit inhibitory concentrations ranging

between 0.1μg/ml and 25μg/ml; 0.1μg/ml and 10μg/ml; 0.5μg/ml and 5μg/ml; 0.5μg/ml and 3μg/ml; and 1.0μg/ml and 10μg/ml, inclusive, in the transmigration assays.

In addition to the above-described antibody inhibitory agents of the invention, the invention also embraces inhibitory agents that are related to, or derived from, the CD47 epitope. According to this aspect of the invention, the above-noted "epitopic" peptides (SEQ. I.D. Nos. 1-35) are provided. The epitopic peptides have sequences which are related to, or derived from, the amino acid sequence of the CD47 epitope to which the C5/D5 antibody binds when CD47, expressed on neutrophils or epithelial cells, is in its native conformation. Thus, the discovery that the CD47 epitope plays an essential role in neutrophil transmigration, i.e., that neutrophil transmigration is "CD47-mediated", suggests that isolated epitopic peptides which mimic the CD47 epitope can be used to identify additional monoclonal antibodies which bind to the CD47 epitope and other agents for detecting CD47 and/or for modulating CD47-mediated transmigration in vivo. The preferred epitopic peptides are selected from SEQ. I.D. Nos. 2-35, more preferably from SEQ. I.D. Nos. 10-23 and 31-33, and most preferably from SEQ. I.D. Nos. 17-23 and 31-33.

According to yet another aspect of the invention, a pharmaceutical composition for modulating an immune response is provided. The composition includes an inhibitory agent that inhibits neutrophil transmigration across a cell layer or through an extracellular matrix, and a pharmaceutically-acceptable carrier. The inhibitory agent is selected from the group consisting of the above-described antibodies which specifically bind to the CD47 epitope and the above-described epitopic peptides. The inhibitory agents are present in the pharmaceutical composition in a therapeutically effective amount, i.e., an amount sufficient to inhibit neutrophil transmigration in vivo. Preferably, the pharmaceutical compositions are packaged to contain sufficient active inhibitory agent for a single dose. In the particularly preferred embodiments, the pharmaceutical composition contains a monoclonal antibody, preferably the C5/D5 antibody, or one or more functionally active fragments thereof. Alternatively, the pharmaceutical compositions contain the above-described antibodies which have the characteristics of the deposited C5/D5 antibody. In yet other embodiments, the pharmaceutical compositions contain the above-described epitopic peptides. The epitopic peptides have an amino acid sequence that is contained within SEQ. I.D. No. 1. Preferably, the epitopic peptides contain between three and twenty amino acids, more preferably between four and twelve amino acids. Exemplary epitopic

peptides include SEQ. I.D. Nos. 2-35. The preferred epitopic peptides contain the minimum sequence SSAKIE (e.g., SEQ. I.D. Nos. 10-23 and 31-33).

In addition to pharmaceutical applications, the antibodies disclosed herein are useful for determining the presence and/or for quantitating the amount of CD47 that is present in a sample. Because CD47 is a component of neutrophils, the antibodies of the invention also are useful for determining the presence or number of neutrophils present in a sample, as well as for labeling CD47 that is expressed on the surface of neutrophils or other cell types (e.g., fibroblasts, red blood cells). Thus, the antibodies of the invention are particularly useful for diagnosing RH null, a condition that is characterized by the absence of RH antigen and greatly diminished CD47 expression on red blood cells. For such diagnostic and research applications, the antibodies of the invention can be incorporated into well-known assay formats (e.g., ELISA, FACS analysis, Western blotting, immunoprecipitation assays) by substituting the antibodies disclosed herein and CD47 (or an epitopic peptide) for the primary antibodies and antigens of the prior art assay formats. Optimization of such assay formats requires no more than routine experimentation by one of ordinary skill in the art.

In another aspect of the invention, a method for inhibiting the migration of a CD47-expressing cell (e.g., a neutrophil) across a cell layer or through an extracellular matrix is provided. Preferably, the method is for inhibiting the migration of neutrophils across a polarized cell layer (e.g., an epithelial cell layer or an endothelial cell layer). The method for inhibiting CD47-expressing cell migration involves contacting at least one of the CD47-expressing cell, the cell layer and the extracellular matrix with an inhibitory agent of the invention (e.g., the above-described antibodies and epitopic peptides). The preferred inhibitory agents of the invention inhibit transmigration in a bidirectional fashion. In a particularly preferred embodiment, the antibody is the deposited C5/D5 antibody or a functionally-active fragment thereof.

In yet another aspect of the invention, a method for modulating an immune response in a subject is provided. The method involves administering to the subject a pharmaceutical composition containing a pharmaceutically-acceptable carrier and one or more of the above-described inhibitory agents of the invention. The inhibitory agent is present in the pharmaceutical composition in a therapeutically effective amount to modulate the immune response. Preferably, the method for modulating an immune response is an improved method which involves inhibiting: (1) the adhesion of neutrophils to the cells of the cell layer and (2) the

transmigration of the neutrophils (or other CD47-expressing cells) across the cell layer or through the extracellular matrix. According to this embodiment, the method involves coadministering the inhibitory agents of the invention (preferably, the above-described antibodies) with "adhesion inhibitory agents" (e.g., other antibodies or antibody fragments) which inhibit adhesion between the neutrophils and the cells in the cell layer. Exemplary adhesion inhibitory agents include antibodies to CD11b, CD11a, ICAM-1 and the selectins (P, E and L selectin). This improved method for modulating an immune response advantageously prevents the initial adhesion of the neutrophils to the cell layer in vivo and inhibits the transmigration of neutrophils which have adhered successfully to the surface cells of the cell layer.

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments and from the accompanying drawings. All references, patents and patent publications identified in this document are incorporated in their entirety herein by reference.

Brief Description of the Drawings

Figure 1 - Functional effects of C5/D5 IgG on PMN transepithelial migration. Figure 1A and 1B represent the inhibitory effects of graded concentrations of C5/D5 IgG for PMN transmigration in the apical-to-basolateral (Ap-Bl, 1A) and basolateral-to-apical (Bl-Ap, 1B) directions. As a binding negative control antibody (CTL), mAb W6/32 was used at 50μg/ml. Migration in the absence of antibody addition (NoAb) is shown in Figure 1A, as is a positive control (transmigration in the presence of 5μg/ml of inhibitory anti-CD11b/CD18 mAb 44a (Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12). mAb 44a inhibited transmigration by 56±3%. In Figures 1C and 1D, T84 monolayers were exposed to 1000U/ml IFNγ or 10 U/ml IL-4 respectively for 48 hours. Following cytokine washout, PMN transmigration assays were performed. For IL-4 experiments, transmigration was in the Ap-Bl direction. For IFNγ experiments, the effects of C5/D5 IgG on transmigration are shown in both directions. In agreement with previous observations (Colgan, S.P. et al. (1993) J. Cell. Biol. 120:785-798), IFNγ pretreatment resulted in diminished physiologically directed transmigration from 63.5±4.4 to 35.6±4.7x10^4 migrated PMN for untreated vs IFNγ - treated T84 monolayers, respectively. In Figure 1E, surface expression of mAb C5/D5 epitope was assayed on control ((-)IFNγ) and IFNγ exposed ((+) IFNγ) T84 cells by ELISA. On the Y axis, surface label represents arbitrary fluorescence units after incubation with FITC conjugated goat anti-mouse IgG.

Figure 2 - Effects of C5/D5 IgG on adhesion. Figure 2A shows neutrophil-T84 adhesion: C5/D5 IgG (25μg/ml) was added to the apical surface of EDTA-treated T84 monolayers followed by the addition of PMN and stimulation with fMLP (n-formyl-Met-Leu-Phe). Adhesion assays were performed as previously described (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479) and in the Examples. As a binding, non-inhibitory control, antibody W6/32 (CTL) was used at the same concentration. Anti-CD11b mAb 44a served as an inhibitory control as described previously (Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12). Adhesion in the absence of antibody is also shown (NoAb). Figure 2B shows the effect of mAb C5/D5 IgG on T84 cell adhesion to purified CD11b/CD18: T84 cells, fluorescently labeled with BCECF-AM (2',7'-bis (2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester Molecular Probes, Inc., Eugene, OR) in the presence or absence of C5/D5 IgG (20μg/ml) or control antibodies, were assayed for adhesion to functionally active CD11b/CD18 in 96-well microtiter plates as described in the Examples. Control antibodies included W6/32 (20μg/ml; negative inhibition), and anti-CD11b mAb 44a (10μg/ml; positive inhibition).

Figure 3 - Alignment of peptide sequences of the C5/D5 antigen with the predicted sequence of CD47. The amino acid sequences obtained from two tryptic peptides of protein immunopurified from C5/D5 IgG are shown in alignment with the predicted extracellular immunoglobulin V- like domain of CD47 (IAP) between residues 60 and 100. The region of homology was expanded from the hypothetical secondary structure as previously reported (Lindberg, F.P. et al. (1993) J Cell Biol. 123:485-96).

Figure 4 - C5/D5 Fab'/F(ab')$_2$ fragments inhibit PMN transepithelial migration. Fab' (25μg/ml) and F(ab')$_2$ (20μg/ml) fragments of C5/D5 IgG were prepared by pepsin digestion and assayed for effects on apical-to-basolateral transmigration using EDTA pre-treated T84 monolayers as described in Figure 1 and in the Examples. As controls, normal mouse IgG1 was pepsin digested in parallel with C5/D5 to make F(ab')$_2$/Fab. In addition, intact C5/D5 (C5/D5 IgG) and W6/32 were used (25μg/ml).

Figure 5 - Relative contributions of neutrophil versus epithelial CD47 to PMN transepithelial migration. Figure 5A shows C5/D5 IgG, at the concentrations in parentheses (μg/ml), preincubated with inverted T84 monolayers (20°C, 1h) before extensive washing followed by immediate use in basolateral-to-apical transmigration assays (see Examples). As a control, monolayers were preincubated with 50μg/ml W6/32 IgG. Figure 5B shows PMN preincubated with equivalent doses of C5/D5 IgG or the binding, non-inhibitory control antibody

W6/32 (25μg/ml) before extensive washing followed by immediate use in basolateral-to-apical transmigration assays. Figure 5C shows collagen coated permeable supports pre-incubated in media overnight that were used in transmigration assays as above except that antibody (10μg/ml) was present in both the upper and lower chambers. Migration is shown in the absence ((-)fMLP) or presence ((+)fMLP) of a 10nM fMLP transwell gradient. In the presence of an fMLP gradient, transmigration in the presence of a control binding antibody W6/32 is compared to that in the presence of C5/D5 IgG.

Figure 6 - C5/D5 IgG inhibits PMN transendothelial migration. Dose responses of C5/D5 IgG on PMN across monolayers of human umbilical vein endothelial cells (HUVECs) were performed exactly as described in Figure 1 and in the Examples. The concentration of antibody in μg/ml is shown in parentheses. As controls, transmigration in the absence of antibody (NoAb) and W6/32 (20μg/ml) are shown.

Figure 7 - Multistep model of neutrophil transepithelial migration. Neutrophil migration across intestinal epithelium naturally occurs in the basolateral-to-apical direction and leads to reversible disruption of tight junctions (denoted by the heavy bar between center and left cell) and ultimately results in collection of PMN on the lumenal (d) surface (termed "crypt abcess" by histopathologists). An initial adhesive event (a) involves PMN adhesion to the epithelial cell (b) basolateral domain and is dependent on CD11b/CD18 (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479; Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12) whereas a subsequent event occurring during migration of PMN between epithelial cells is dependent on CD47 (c).

Detailed Description of the Invention

While not intending to be bound by any particular theory, it is believed that the novel compositions disclosed herein modulate a mucosal immune response by inhibiting transmigration of neutrophils (PMN) across a polarized epithelial cell layer in vivo. Accordingly, the experiments described herein were directed to: (1) identifying monoclonal antibody reagents which inhibit neutrophil transmigration across an epithelial cell monolayer; and (2) identifying the antigens and in particular, the epitopes of said antigens to which the monoclonal antibody reagents of the invention specifically bind as an essential step in inhibiting PMN transmigration.

Antibodies and Related Inhibitory Agents of the Invention

The monoclonal antibodies of the invention were prepared by immunizing mice with membranes derived from a model polarized human intestinal epithelium and were characterized

with respect to their functional activities by observing the effects of the antibodies on epithelial-PMN interactions (e.g., adhesion interactions, transmigration interactions). The preparation and characterization of a particularly preferred antibody, the "C5/D5" antibody having ATCC Accession No. HB-12021 (referred to herein as "C5/D5") is described in the Examples. The C5/D5 antibody inhibits PMN transepithelial migration but does not inhibit either the initial adhesion of PMN to epithelial cells or the adhesion of epithelial cells to purified CD11b/18.

Characterization of the C5/D5 antibody with respect to epitope specificity, e.g., by microsequencing and cross blotting/ELISA assays, demonstrated that the C5/D5 antigen is an immunoglobulin family member known as CD47. Further results demonstrated that CD47 is expressed on colonic epithelium and also on PMN. In view of these discoveries, it is believed that CD47 plays an essential role in the transmigration of PMN from the epithelial basolateral surface to the intestinal lumen. Moreover, in view of a possible role for CD47 in modulating $\alpha_v \beta_3$ function, we believe that agents which modulate CD47-mediated events (e.g., the antibodies and epitopic peptides of the invention) also can be used to modulate $\alpha_v \beta_3$-mediated functions such as angiogenesis and tumor metastasis. Accordingly, in a broad aspect, the invention relates to antibodies and related inhibitory agents which modulate a variety of CD47-mediated events, including CD47-mediated PMN transmigration across a cell layer or through an extracellular matrix in vivo or in vitro and CD47-mediated integrin functions (e.g., β_1-integrin functions).

Although the following description is directed to a preferred embodiment of the invention, namely, antibody compositions and their methods of use for inhibiting PMN transmigration across an epithelial cell monolayer, it should be understood that this description is illustrative only and is not intended to limit the scope of the instant invention. Thus, in its broadest sense, the invention relates to the discovery that a monoclonal antibody (the "C5/D5 antibody") which binds to a particular epitope on CD47 (the "CD47 epitope") is capable of inhibiting (i.e., reducing to a statistically significant extent) PMN migration across a cell layer. The compositions and methods disclosed herein also are useful for identifying additional antibody and related reagents (e.g., epitopic peptides which mimic the CD47 epitope) that are capable of inhibiting PMN transmigration in vivo or in vitro.

Agents which modulate transmigration of PMN across a cell layer (e.g., an epithelial or endothelial cell layer) or through an extracellular matrix are useful for treating autoimmune diseases that are characterized by lymphocyte accumulation at epithelial sites (e.g., ulcerative colitis, Crohn's disease, celiac disease, sarcoidosis, psoriasis, the late phase component of

asthma, contact dermatitis, scleroderma and graft versus host disease). Such agents also are useful for targeting the delivery of therapeutic and/or diagnostic agents to cells which express CD47 (e.g., neutrophils, epithelial cells, endothelial cells, fibroblasts, red blood cells), thereby permitting the design of more appropriate therapies for treating infectious diseases of epithelial sites (e.g., pulmonary tuberculosis, leprosy, cutaneous leishmaniosis, and parasitic or viral infectious diseases of the intestinal tract) by affecting the expression and/or function of the targeted cells.

According to one aspect of the invention, an antibody that binds to the CD47 epitope and inhibits PMN transmigration across a cell layer is provided. Preferably, the antibody is a monoclonal antibody (e.g., a mouse, chimeric or humanized monoclonal antibody) which specifically recognizes and binds to the CD47 epitope. The hybridoma cell line expressing the preferred monoclonal antibody, the C5/D5 monoclonal antibody, was deposited at the ATCC, Rockville, MD, on January 17, 1996 and accorded ATCC Accession No. HB-12021.

The term "antibody" is a term of art which means an immunoglobulin molecule or a fragment immunoglobulin molecule having the ability to specifically bind to a particular antigen. The term "antibody" as used herein means not only intact antibody molecules but also functionally-active fragments of antibody molecules, i.e., fragments which retain antigen binding ability. The preferred embodiments of the invention are directed to the C5/D5 antibody and functionally-active fragments of this deposited antibody. Functionally-active antibody fragments contain the antigen-binding region ("paratope") of the intact antibody. Accordingly, the functionally active antibody fragments of the invention specifically bind to the CD47 epitope and preferably, also exhibit a further functional activity of the C5/D5 antibody (e.g., the ability to inhibit neutrophil transmigration in a neutrophil transmigration assay). Exemplary functionally-active antibody fragments include an $F(ab')_2$ fragment, an F(ab') fragment, an Fv fragment and an Fd fragment. The preparation of antibody fragments is routine in the art (see, e.g., the Examples which describe the preparation and testing of $F(ab')_2$ and F(ab') fragments).

The term "paratope" is a term of art which refers to the portion of an antibody which specifically binds to an epitope in the antigen. A paratope is composed of amino acid sequences in both the immunoglobulin heavy and light chains (see, e.g., Clark, W.R. (1986) The Experimental Foundations of Modern Immunology, Wiley and Sons, Inc., New York, New York; Roitt I. (1991) Essential Immunology, 7th Edition, Blackwell Scientific Publications, Oxford). Thus, the paratope of the C5/D5 antibody, or functionally-active fragments thereof, which bind

to the above-described CD47 epitope and which result in inhibition of PMN transmigration, is defined by the amino acid sequences of the immunoglobulin heavy and light chain V regions. The nucleic acid sequences encoding these amino acid sequences can be identified in accordance with standard procedures, e.g., by sequencing from both the 5' and 3' of the Fd heavy chain fragment or the light chain. Due to the degeneracy of the DNA code, multiple nucleic acid sequences can encode the particular amino acid sequences which form the paratope of the C5/D5 antibody. Accordingly, the instant invention embraces not only the antibodies and antibody fragments which directly inhibit PMN transmigration, but also the nucleic acid sequences which encode such antibodies and fragments, vectors containing these nucleic acids, and cells containing the vectors or isolated nucleic acids which encode the antibodies and functionally-active antibody fragments of the invention.

In general, intact antibodies are said to contain "Fc" and "Fab" regions. The Fc regions are involved in complement activation and are not involved in antigen binding. An antibody from which the Fc' region has been enzymatically cleaved, or which has been produced without the Fc' region, designated an "$F(ab')_2$" fragment, retains both of the antigen binding sites of the intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an "Fab'" fragment, retains one of the antigen binding sites of the intact antibody. Fab' fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain, denoted "Fd." The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity). Isolated Fd fragments retain the ability to specifically bind to antigen epitopes.

Within the antigen-binding region of an antibody are complementarity determining regions (CDRs) which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, A.G. Clark, 1986 supra; Roitt, 1991 supra). In both the heavy chain Fd fragment and the light chain of the IgG immunoglobulins, there are four framework regions (FR1-FR4) separated respectively by three complementarity determining regions (CDR1-CDR3). The CDRs, and in particular the CDR3 region, and more particularly the heavy chain CDR3, are primarily responsible for antibody specificity.

The complete amino acid sequences of the antigen-binding Fab' portion of the C5/D5 monoclonal antibodies, as well as the relevant FR and CDR regions, can be determined using

amino acid sequencing methods that are routine in the art. It is well established that non-CDR regions of a mammalian antibody may be replaced with corresponding regions of non-specific or hetero-specific antibodies while retaining the epitope specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc′ regions to produce a functional antibody. Thus, for example, PCT International Publication No. WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

The present invention also provides the $F(ab')_2$, Fab, Fv and Fd fragments of the C5/D5 monoclonal antibody; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of the C5/D5 antibody have been replaced by homologous human or non-human sequences; chimeric $F(ab')_2$ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of the C5/D5 antibodies have been replaced by homologous human or non-human sequences; chimeric Fab' fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR 1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. Thus, one of ordinary skill in the art may alter the C5/D5 antibody by the construction of CDR grafted or chimeric antibodies or antibody fragments containing, all or part thereof, of the heavy and light chain V-region CDR amino acid sequences for the deposited antibody (see, e.g., Jones et al., Nature 321:522 (1986); Verhoeyen et al., Science 39:1534 (1988) and Tempest et al., Biotechnology 9:266 (1991), without destroying the specificity of the antibodies for the CD47 epitope. Such CDR grafted or chimeric antibodies or antibody fragments can be effective in inhibiting PMN migration across a cell layer. Such chimeric antibodies and functionally-active antibody fragments of the invention have the characteristics of the C5/D5 antibody.

Preferably, the chimeric antibodies of the invention are fully human monoclonal antibodies which include at least the heavy chain CDR 3 region of the C5/D5 antibody. Such chimeric antibodies may be produced in which some or all of the FR regions of C5/D5 have been replaced by other homologous human FR regions. In addition, the Fc portions may be replaced

so as to produce IgA or IgM as well as IgG antibodies bearing some or all of the CDRs of the C5/D5 antibody. Of particular importance is the inclusion of the C5/D5 heavy chain CDR3 region, and to a lesser extent, the other CDRs of C5/D5. Such fully human chimeric antibodies are particularly preferred in that they do not evoke an immune response.

For inoculation or prophylactic uses, the antibodies of the present invention are preferably intact antibody molecules which include the Fc region. Such intact antibodies will have longer half-lives than smaller fragment antibodies, e.g., Fab' fragments, and are more suitable for intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous or transdermal administration. For topical administration (e.g., administration to the lumenal lining of the lungs as by aerosol) Fab' fragments, including chimeric Fab' fragments, are preferred. Fab' fragments offer several advantages over $F(ab')_2$ and whole immunoglobulin molecules for topical application. For example, because Fab' fragments have only one binding site for their cognate antigen, the formation of immune complexes is precluded. Further, because Fab' fragments lack an Fc region, an adverse inflammatory reaction which is Fc-mediated cannot be triggered. Moreover, the tissue penetration of smaller Fab' fragments is likely to be significantly greater than that of a larger molecule. In addition, Fab' fragments can be produced inexpensively in bacterial culture in large quantities.

Smaller antibody fragments and epitope-binding peptides having binding specificity for the CD47 epitope which can be used to inhibit PMN (or other CD47-expressing cell) transmigration across a cell layer or through an extracellular matrix also are embraced within the present invention. For example, single-chain antibodies can be constructed in accordance with the methods described in U.S. Patent No. 4,946,778 to Ladner et al. Such single-chain antibodies include the variable regions of the light and heavy chains joined by a flexible linker moiety. Methods for obtaining a single domain antibody ("Fd") which comprises an isolated VH single domain, also have been reported (see, for example, Ward et al., Nature 341:644-646 (1989)).

Using routine procedures known to those of ordinary skill in the art, one can determine whether an altered or chimeric antibody has the same specificity as the C5/D5 antibody of the invention by determining whether the altered or chimeric antibody blocks the C5/D5 antibody from binding to CD47 or more preferably, from binding to the CD47 epitope. If the putative monoclonal antibody having the "characteristics" of the C5/D5 antibody competes with the C5/D5 antibody, as shown by a decrease in binding of the C5/D5 antibody to the antigen, then

one can conclude that the putative antibody or putative functionally-active antibody fragment bind to the same or a closely related epitope.

The C5/D5 antibody can be used to produce anti-idiotypic antibodies which can be used to identify novel hybridomas having the same binding specificity as C5/D5. In addition, such anti-idiotypic antibodies can be used for active immunization (Herlyn, et al., Science 232:100, 1986). Anti-idiotypic antibodies can be produced using well known hybridoma techniques (Kohler and Milstein, Nature, 256:495, 1975). Anti-idiotypic antibodies can be prepared by immunizing an animal with C5/D5 or an antibody having the structural characteristics of the C5/D5 antibody disclosed herein. The anti-idiotypic antibodies which are produced in the immunized animal are specific for the monoclonal antibodies of the invention and hence, can be used to identify other hybridomas with the same idiotype (i.e., antigen binding site) as the C5/D5 antibody (or related antibody used for the immunization).

In a particularly preferred embodiment, the antibody is the C5/D5 antibody having ATCC Accession No. HB-12021. In contrast to conventional immunization protocols (which involve immunizing an animal with whole cells or purified antigen and selecting for adhesion to a cell which expresses the antigen), the C5/D5 antibody was obtained by immunizing animals with epithelial membrane fragments and screening the hybridomas for a functional activity, namely, the ability to inhibit neutrophil transmigration. The resultant monoclonal antibodies were exceptionally potent inhibitors of neutrophil transmigration. The successful preparation of novel monoclonal antibodies which inhibit PMN transmigration across the epithelial monolayer suggests that (1) the selection of epithelial membrane fragments as the immunogen and (2) the functional activity screening assay were critical to the successful preparation of an antibody having the inhibitory activity of the C5/D5 antibody.

The C5/D5 antibody specifically binds to the CD47 epitope, a portion of the CD47 extracellular region. The amino acid sequence for the CD47 antigen is identified in GenBank Accession No. S36644. For consistency, the amino acid residue numbers for CD47 that are used throughout this document are based upon the numbering system used in GenBank Accession No. S36644. The results presented herein indicate that the epitope is located in the CD47 extracellular region which contains the IgV-like domain, and is more particularly located to the region defined by amino acid residues 60 to 100, inclusive (SEQ. I.D. No. 2). It is believed that the CD47 epitope contains at least one sequence selected from the group consisting of SEQ. I.D. Nos. 2-35. Preferably, the epitope contains a sequence that is selected from the group consisting

of SEQ. I.D. Nos. 10-23 and 31-33. More preferably, the epitope includes at least the amino acid sequence SSAKIE (SEQ. I.D. No. 17) and optionally, includes up to an additional three amino acids on each side of this sequence (see, e.g., SEQ. I.D. Nos. 18-23). In a particularly preferred embodiment, the epitope includes the amino acid sequence of SEQ. I.D. No. 31 and optionally, includes up to an additional three amino acids on each side of this sequence (see, e.g., SEQ. I.D. Nos. 32-33). Thus, by identifying an antibody which inhibits PMN transmigration across an epithelial cell monolayer, and further, by identifying the epitope for this antibody (a specific region within the CD47 extracellular domain), Applicants have taught that which is essential for one of ordinary skill in the art to prepare epitopic peptides and antibodies to this region (and to the epitopic peptides) which inhibit PMN transmigration across a cell layer.

According to yet another aspect of the invention, antibodies having the "characteristics" of the monoclonal antibody having ATCC Accession No. HB-12021 (C5/D5 antibody) are provided. As used herein, "characteristics" refers to the distinct structural and functional properties of the C5/D5 antibody which confer upon it the ability to (1) bind to the CD47 epitope and (2) inhibit PMN transmigration across a cell layer. Monoclonal antibodies having the characteristics of the C5/D5 antibody share both structural (e.g., epitope specificity, paratope sequence) and functional similarities (e.g., a transmigration inhibitory activity) with the deposited C5/D5 antibody. Thus, for example, an exemplary structural characteristic of the C5/D5 antibody is the specificity of the antibody for binding to the CD47 epitope, i.e., the antibodies and antibody fragments of the invention have an antigen-binding region which binds to substantially the same epitope on the CD47 antigen to which the C5/D5 antibody binds. By "substantially the same" it is meant that the epitope includes the minimum amino acid sequence that is specifically recognized by the C5/D5 antibody but may optionally contain additional amino acids, the inclusion of which does not inhibit binding of the antibody to its antigen. In the particularly preferred embodiments, the antibodies of the invention specifically bind to the identical epitope on CD47 to which the C5/D5 antibody binds (i.e., the "CD47 epitope"). Preferably, the CD47 epitope is defined by an amino acid sequence (containing between three and twenty amino acids) located within SEQ. I.D. No. 1. In the particularly preferred embodiments, the antibodies and antibody fragments of the invention specifically bind to a minimum amino acid sequence containing SSAKIE (SEQ. I.D. No. 17). Additional sequences which contain this minimum amino acid sequence are provided in SEQ. I.D. Nos. 2, 10-16, 18-23 and 31-33. Thus, monoclonal antibodies having the characteristics of the monoclonal having

ATCC Accession No. HB-12021 are antibodies which specifically bind to the CD47 epitope or to epitopic peptides derived from the CD47 epitope.

The antibodies of the invention also can be defined in terms of antibody structure, i.e., by defining the antibody paratope. Thus, the amino acid sequence of the C5/D5 paratope can be used to define alternative "monoclonal antibodies having the characteristics" of the monoclonal antibody having ATCC Accession No. HB-12021. The amino acid sequence defining the C5/D5 antibody paratope can be determined using routine amino acid sequencing procedures. Once the particular amino acid sequence defining the antigen binding region is determined, this sequence can be incorporated into other chimeric molecules, or alternatively, can be used alone to form novel agents for inhibiting PMN transmigration across a cell layer or through an extracellular matrix. Thus, antibodies having the characteristics of the C5/D5 antibody have paratopes which are identical or substantially identical to the paratope of the C5/D5 antibody. By "substantially identical" it is meant that the amino acid sequence of the paratope may include conservative amino acid substitutions which do not adversely affect the ability of the antibody to bind to the CD47 epitope and inhibit PMN transmigration across a cell layer (e.g., as measured in a screening assay such as that described in the Examples). As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative size or charge characteristics of the peptide in which the amino acid substitution is made. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) MILV; (b) FYW; (c) KRH; (d) A; (e) ST; (f) QN; and (g) ED.

The phrase "monoclonal antibodies having the characteristics of the C5/D5 antibody" embraces antibodies having inhibitory concentrations which are substantially identical (i.e., statistically within the assay margin of error) or within two standard deviation of the inhibitory concentration of the C5/D5 antibody. An exemplary assay for measuring the ability of a putative antibody of the invention to inhibit PMN transmigration across a cell layer is provided in the Examples. The exemplary assay is predictive of the ability of an antibody to inhibit transmigration in vivo and hence, can be used to select antibodies and/or antibody fragments for therapeutic applications, as well as diagnostic and research applications. The transmigration assay measures the transmigration of neutrophils across a cell layer (e.g., an epithelial or endothelial cell layer) or a cellular filter (e.g., a Boyden chamber) or through a matrix. The antibodies of the invention exhibit an inhibitory concentration (between 0.1 μg/ml and 50 μg/ml, inclusive) in a neutrophil transmigration assay to result in at least between about 65-75%

inhibition of neutrophil migration in the assay. In the particularly preferred embodiments, the antibody has an inhibitory concentration between 0.5 μg/ml and 3 μg/ml. In general, the antibodies of the invention have an inhibitory concentration between 1.0 μg/ml and 10 μg/ml, inclusive. Preferably, the inhibitory concentration which results in at least 75% inhibition in the assay is between 0.1 μg/ml and 25 μg/ml; more preferably, between 0.1 μg/ml and 10 μg/ml; and most preferably between 0.5 μg/ml and 5 μg/ml, inclusive. With respect to the in vitro transmigration assay disclosed in the Examples, an antibody having the characteristics of the C5/D5 antibody preferably has an inhibitory concentration which is between about 0.5 μg/ml and 5 μg/ml, inclusive, to result in at least about 65-75% inhibition. Although the C5/D5 antibody exhibits an inhibitory concentration in this assay of about 0.75 μg/ml, one skilled in the art can use the assay to select hybridomas having a range of inhibitory activities. Thus, using no more than routine skill in the art, alternative antibodies which exhibit inhibitory concentrations in a range which embraces the C5/D5 antibody inhibitory concentration can be identified. In a preferred embodiment, the antibody having the characteristics of the C5/D5 antibody has an inhibitory concentration which results in at least 80%; more preferably, at least 85% and most preferably, at least 90% inhibition (at the above-described inhibitory concentrations) in the transmigration assay.

In the particularly preferred embodiments, the monoclonal antibodies having the characteristics of the C5/D5 antibody have substantially identical inhibitory concentrations to the C5/D5 antibody in the exemplary transmigration assay provided herewith and bind to the same epitope on CD47 to which the C5/D5 antibody binds (i.e., the CD47 epitope). Of course, alternative antibodies can be selected which bind to the CD47 epitope and which further have greater inhibitory activity with respect to the C5/D5 antibody. Such antibodies also are embraced within the invention. In the preferred embodiments, the antibodies "having the characteristics of the C5/D5 antibody" inhibit transmigration of PMN in a bidirectional fashion, preferably across a polarized cell monolayer. More preferably, the antibodies of the invention do not inhibit CD11b/CD18-mediated adhesion of the PMN to the cell layer or to isolated cells of the cell layer. Rather, the antibodies of the invention preferably inhibit transmigration of the PMN and do not participate in inhibiting adhesion of PMN to the cells of the cell layer.

According to another aspect of the invention, a pharmaceutical composition is provided. The pharmaceutical composition contains an inhibitory agent that inhibits transmigration of a CD47-expressing cell (e.g., a neutrophil) across a cell layer and/or through an extracellular

matrix and a pharmaceutically-acceptable carrier. Two general categories of such inhibitory agents are embraced within the instant invention: (1) antibodies or functionally-active antibody fragments that are related to, or derived from, the deposited C5/D5 antibody and (2) epitopic peptides (discussed below) that are related to, or derived from, the CD47 epitope. The inhibitory agent is present in the preparation in a therapeutically-effective amount, i.e., an amount which is capable of inhibiting CD47-expressing cell (e.g., neutrophil) transmigration in vivo. Such amounts and dosages can be determined in accordance with standard practice taking into account the particular weight, age and other characteristics of the recipient to which the pharmaceutical composition is to be administered.

Preferably, the agent of the pharmaceutical composition is a monoclonal antibody, more preferably, the agent is the monoclonal antibody having ATCC Accession No. HB-12021. Alternatively, the agent is an antibody fragment such as the above-described functionally-active C5/D5 antibody fragments (e.g., $F(ab')_2$, Fab, Fv, Fd). Alternative monoclonal antibodies which serve as inhibitory agents are chimeric antibodies containing at least one of the functionally-active fragments disclosed herein. Regardless of the source of the monoclonal antibody or fragment, it is essential that the antibody/antibody fragment inhibitory agent be capable of: (1) specifically binding to the CD47 epitope, and (2) specifically inhibiting neutrophil transmigration across a cell layer, e.g, as measured in an in vitro transmigration assay such as the assay described in the Examples.

In general, pharmaceutically-acceptable carriers for monoclonal antibodies, antibody fragments and peptides are well-known to those of ordinary skill in the art. As used herein, a pharmaceutically-acceptable carrier means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients, i.e., the ability of the inhibitory agent to inhibit PMN transmigration. The term "physiologically-acceptable" refers to a non-toxic material that is compatible with the biological systems such as a cell, cell culture, tissue or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers and other materials which are well-known in the art. Exemplary pharmaceutically acceptable carriers for peptides in particular are described in U.S. Patent No. 5,211,657. The peptides of the invention may be formulated into preparations in solid, semi-solid, liquid or gaseous forms such as tablets, capsules, powders, granules, ointments, solutions, depositories, inhalants and injections, and usual ways for oral, parenteral or surgical

administration. The invention also embraces locally administering the compositions of the invention as implants.

The inhibitory agents of the invention are useful as therapeutics to inhibit PMN transmigration across epithelial cell layers in vivo. According to this embodiment, the antibodies, antibody fragments, or other inhibitory peptides and nucleic acids of the invention are used in a therapeutically effective amount, i.e., an amount which is sufficient to inhibit PMN transmigration to an extent which will prevent or reduce the migration of PMN across a cell layer or through an extracellular matrix. In general, the therapeutically effective amount of the antibody, antibody fragments or peptides may vary with the recipient's age, condition and sex, as well as the extent of the disease state in the subject and can be determined by a physician of ordinary skill in the art. The dosage may be adjusted by the individual physician or veterinarian in the event of complications. A therapeutically effective amount can vary from about 0.01 mg/kg to about 500 mg/kg, preferably from about 0.1mg/kg to about 200 mg/kg, most preferably from about 0.2mg/kg to about 20mg/kg, in one or more dose administrations daily, for one or several days.

The antibodies or antibody fragments of the invention can be administered by injection or by gradual infusion over time. The administration of the antibodies of the invention may, for example, be intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous or transdermal. Those of skill in the art can readily determine the various parameters for preparing these alternative pharmaceutical compositions without resort to undue experimentation. Preparations for parenteral administration includes sterile aqueous or nonaqueous solutions, suspensions and emulsions. Examples of nonaqueous solvents are propylene glycol, polyethylene glycol, vegetable oil such as olive oil, an injectable organic esters such as ethyloliate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

The antibodies and antibody fragments of the invention can be used for therapeutic, diagnostic and research applications. According to one aspect of the invention, a method for modulating an immune response in a subject is provided. The method involves administering to

the subject the above-described pharmaceutical composition. The antibody, fragment or epitopic peptide is present in the composition in a therapeutically effective amount to modulate (i.e., reduce or prevent the immune response). In a particularly preferred embodiment, the antibodies of the invention are coadministered with an "adhesion inhibitory agent," i.e., an agent which inhibits adhesion between the PMN and the cells in the cell layer through which the PMN transmigrates. Coadministration of the antibodies of the invention with one or more adhesion inhibitory agents, such as an antibody to CD11b, CD11a, ICAM -1 or a selectin (e.g., P, E and/or L selectin) results in an improved method for modulating an immune response in which both adhesion of the PMN to the cell layer and transmigration of the PMN across the cell layer and/or extracellular matrix are inhibited.

The antibodies of the invention can be used to inhibit the migration of PMN across a cell layer (e.g., a polarized cell monolayer of epithelial cells) or through an extracellular matrix in vivo or in vitro. The antibodies also can be used to inhibit PMN migration across layers of endothelial cells, epithelial cells, mesenchymal cells (e.g., fibroblasts, stomal cells) and extracellular matrix components (e.g., laminin, fibronectin, entactin, proteoglycans, collagen). The method for inhibiting PMN migration involves contacting at least one of the PMN, the cell layer and the extracellular matrix with an antibody of the invention prior to initiating transmigration. In the preferred embodiments, the method for inhibiting migration of PMN is directed to inhibiting migration in a bidirectional fashion across a polarized cell monolayer. Preferably, the antibody is the C5/D5 antibody. Alternatively, the antibody used for this purpose is an antibody or antibody fragment having the above-described characteristics of the C5/D5 antibody, i.e., the above-described structural and functional properties of the C5/D5 antibody.

The antibodies of the invention can be used to measure the amount of CD47 in a biological sample or in a standard sample in an assay kit for determining the presence, absence, or for quantitating the amount of CD47 in a sample. Additional in vitro assays employing the monoclonal antibodies of the invention to measure CD47 include ELISA assays, FACS analysis, Western blotting. The antibodies and fragments of the invention also can be used to visualize expression of the CD47 epitope on PMN, epithelial and other cell types (e.g., by attaching a label, such as a radioactive, enzyme or fluorescent tag, to the antibody or peptide and allowing the antibody or fragment to contact and specifically bind to the CD47 epitope in vivo and/or in vitro. Methods for coupling such toxins and/or agents to antibodies and/or peptides for in vivo and in vitro applications are disclosed in, for example, Killen and Lindstrom (1984), J. Immun.

133:1335; Jansen, F.K., et al. (1982), Immunolog. Rev. 62:185-216. See also U.S. Patent Nos. 3,652,761; 4,478,946 and 4,554,088.

The monoclonal antibodies of the invention also are useful in screening assays for identifying pharmaceutical lead compounds in molecular or phage libraries. See, e.g., U.S. Patent No. 5,010,175 issued to Rutter et al. A "molecular library" refers to a collection of structurally-diverse molecules. Molecular libraries can be chemically-synthesized or recombinantly-produced. As used herein, a "molecular library member" refers to a molecule that is contained within the molecular library. Accordingly, screening refers to the process by which library molecules (e.g., "epitopic" peptides) are tested for the ability to modulate neutrophil transmigration across a cell layer. As used herein, a "pharmaceutical lead compound" refers to a molecule which is capable of modulating neutrophil transmigration across a cell layer. Thus, transmigration screening assays are useful for assessing the ability of a library molecule to inhibit the transmigration of a neutrophil across a cell layer in vivo or in vitro.

Libraries of molecularly diverse molecules can be prepared using chemical and/or recombinant technology. Such libraries for screening include recombinantly-produced libraries of fusion proteins. An exemplary recombinantly-produced library is prepared by ligating fragments of the cDNA for the CD47 epitope into, for example, the pGEX-2T vector (Pharmacia, Piscataway, NJ). This vector contains the carboxy terminus of glutathione S-transferase (GST) from Schistosoma japonicum. Use of the GST-containing vector facilitates purification of GST-CD47 epitope fusion proteins from bacterial lysates by affinity chromatography on glutathione sepharose. After elution from the affinity column, CD47 epitope fusion proteins are tested for activity by, for example, contacting at least one fusion protein with a neutrophil prior to (or concurrently with) contacting the neutrophil with the cell layer of the transmigration assay. Fusion proteins which inhibit transmigration of the neutrophil across the cell layer are selected as pharmaceutical lead compounds and/or to facilitate further characterization of the CD47 epitope. See, for example, Koivunen E. et al. (1993) J. Biol. Chem. 268(27):20205 which describes the selection of peptides which bind to the $\alpha^5\beta_1$ integrin from a phage display library. In this manner, the precise amino acid sequence which defines the CD47 epitope can be determined (see also the Examples for an exemplary protocol for determining epitope amino acid sequences.)

Antibody-based screening assays are performed by, for example, contacting an antibody (that specifically binds to the CD47 epitope and preferably inhibits neutrophil transmigration across a cell layer) with a CD47-expressing cell (e.g., a neutrophil) in the presence and absence of at least one member of the molecular library and determining whether the library member modulates antibody binding and transmigration of the neutrophil across a cell layer in the assay. In a particularly preferred embodiment, the antibody-based screening assay involves: (1) performing a first transmigration assay in the absence of the library molecule to obtain a first antibody assay result; (2) performing a second transmigration assay in the presence of the library molecule to obtain a second assay result; and (3) comparing the first and the second assay results to determine whether the molecular library member modulates neutrophil migration across a cell layer. According to this embodiment, a second assay result which shows increased neutrophil transmigration indicates that the library member has an inhibitory activity with respect to the antibody. Transmigration assays also can be used to assess the relative inhibitory concentrations of a molecular library member or antibody/antibody fragment in a transmigration assay and to identify those inhibitory agents which inhibit transmigration by at least, e.g., 75%. In an analogous manner, transmigration assays can be used to assess the relative affinity of an antibody of the invention for a library member to further identify the amino acids/amino acid sequences that are important to CD47 antigen binding.

According to yet another aspect of the invention, as isolated epitopic peptide to which the C5/D5 antibody specifically binds, and optionally, which is capable of inhibiting neutrophil transmigration across a cell layer is provided. The isolated epitopic peptides of the invention are related to, or derived from, a portion of the extracellular domain of CD47 (SEQ. I.D. No. 1). More particularly, the epitopic peptides are related to, or derived from amino acids 60 to 100 of CD47 (SEQ. I.D. No. 2). Exemplary epitopic peptides are provided in SEQ. I.D. Nos. 3-35.

The term "isolated" in reference to the inhibitory agents of the invention, means that the peptides of the invention are essentially free of other substances with which they may be found in nature to an extent that is practical and appropriate for their intended use. In particular, the peptides are sufficiently pure and are sufficiently free from other biological substituents of their host cells so as to be useful in, for example, sequencing or producing pharmaceutical preparations. Using techniques known in the art, isolated peptides can be produced which are based upon the amino acid sequences of the proteins from which they are derived. Alternatively,

isolated peptides can be produced having sequences which are deduced from the nucleic acid sequence which encodes the protein from which they are derived. An isolated peptide of the invention may be admixed with a pharmaceutically-acceptable carrier in a pharmaceutical composition. However, the peptide is nonetheless isolated in that it has been substantially separated from the substances with which it may be associated in living systems.

As used herein in reference to a peptide (e.g., an antibody fragment or epitopic peptide), the term "isolated" embraces a cloned expression product of an oligonucleotide; a peptide which is isolated following cleavage from a larger polypeptide; or a peptide that is synthesized, e.g., using solution and/or solid phase peptide synthesis methods as disclosed in, for example, U.S. 5,120,830. Accordingly, the phrase "isolated peptides" embraces peptide fragments of the C5/D5 antibody, functionally equivalent peptide analogs of the antibody fragments, and epitopic peptides.

As used herein, the term "peptide analog" refers to a peptide which shares a common structural feature with the molecule to which it is deemed to be an analog. A "functionally equivalent" peptide analog is a peptide analog which further shares a common functional activity with the molecule to which it is deemed an analog. Thus, a "functionally equivalent epitopic peptide analog" refers to a peptide analog that is specifically bound by the C5/D5 antibody and that optionally, is capable of inhibiting the transmigration of PMN across a cell layer. Similarly, a "functionally equivalent antibody peptide analog" refers to an antibody peptide analog that specifically binds to the CD47 epitope and optionally, inhibits PMN transmigration. Functionally equivalent antibody peptide analogs are identified, for example, in in vitro transmigration assays (see, e.g., the transmigration assay provided in the Examples) that measure the ability of the antibody peptide analog to inhibit PMN transmigration across a cell layer. Such assays are predictive of the ability of a molecule to inhibit this transmigration in vivo. Accordingly, a "functionally equivalent antibody peptide analog" of the C5/D5 antibody includes structural variants of the C5/D5 paratope, and fragments of the C5/D5 antibody which specifically bind to the CD47 antigen, provided that the antibody peptide analogs inhibit PMN transmigration across a cell layer. The preferred peptide analogs are structural variants in which the interamino acid peptide bond has been replaced by a linkage which is not susceptible to proteolytic cleavage, e.g. an ester or ether linkage. (See, e.g., March, J., Advanced Organic Chemistry, 4th Ed., New York, NY, Wiley and Sons, 1985), pp.326-1120). The preparation of such structural variants is well-known to those of ordinary skill in the art.

The antibodies, antibody fragments and epitopic peptides of the invention include "unique fragments" which are related to, or derived from, the antigen binding portion of the C5/D5 antibody (for antibody-inhibitory agents) or are related to, or derived from, the CD47 epitope (for epitopic peptides). A "unique fragment" of a protein or nucleic acid is a fragment which is not currently known to exist elsewhere in nature except in allelic or allelomorphic variants. Unique fragments act as a "signature" of the gene or protein from which they are derived. The unique fragment will generally exceed 15 nucleotides or 5 amino acids in length. One of ordinary skill in the art can readily identify unique fragments by searching available computer databases of nucleic acid and protein sequences such as Genbank, (Los Alamos National Laboratories, U.S.A.), EMBL or SWISS-PROT. A unique fragment is particularly useful, for example, in generating other monoclonal antibodies (e.g., to the CD47 epitope or anti-idiotypic antibodies to the C5/D5 paratope) or in screening genomic DNA or cDNA libraries.

It will be appreciated by those skilled in the art that various modifications of the foregoing peptides can be made without departing from the essential nature of the invention. Accordingly, it is intended that the antibodies as well as the peptides of the invention can include conservative amino acid substitutions which do not adversely affect the ability of the antibodies and peptides to, for example, bind to the CD47 epitope or inhibit PMN transmigration. Thus, for example, antibodies and peptides of the invention which are coupled to a solid support (such as a polymeric bead for use, e.g., in an ELISA or other amino assay), a carrier molecule (such as keyhole limpit hemocyanin for enhancing an immune response to the peptide), a toxin (such as ricin) or a reporter group (such as a radiolabel or other tag), also are embraced within the teachings of the invention.

An exemplary protocol for developing hybridomas which specifically bind to the CD47 epitope and prevent PMN transmigration across a cell monolayer is illustrated in the Examples. It has been discovered, surprisingly, that monoclonal antibodies having the above described transmigration inhibitory characteristics can be prepared by (1) using epithelial cell membranes as the immunogen and (2) selecting for antibodies which inhibit a transmigration function. Previous efforts to prepare antibodies to the CD47 antigen employed either isolated CD47 as the immunogen or whole cells and selected antibodies based upon their ability to bind to CD47. Because the selection criteria (a functional assay) used herein differ from those used in the prior art for antibody preparation, it is believed that the prior art antibodies recognize CD47 at sites distinct from the CD47 epitope defined herein and hence, are incapable of inhibiting CD47

mediated PMN transmigration across a cell layer. Thus, the selection of the immunogen used for preparing the C5/D5 hybridoma, together with a screening assay in which antibodies are selected on the basis of a transmigration inhibitory activity, represent critical aspects in the successful preparation of a monoclonal antibody that is capable of inhibiting CD47 mediated PMN transmigration across a cell monolayer.

Nucleic Acids of the Invention

The invention also provides isolated oligonucleotides that encode the antibodies, functionally active antibody fragments and epitopic peptides of the invention and functionally equivalent peptide analogs thereof.

As used herein, the term "isolated" in reference to an oligonucleotide, means an RNA or DNA polymer, portion of genomic nucleic acid, cDNA or synthetic nucleic acid which, by virtue of its origin or manipulation: a) is not associated with all of a nucleic acid with which it is associated in nature (e.g., is present in a host cell as a portion of an expression vector); or b) is linked to a nucleic acid or other chemical moiety other than that to which it is linked in nature; or c) does not occur in nature. By "isolated" it is further meant a nucleic acid sequence: i) amplified in vitro by, for example, the polymerase chain reaction (PCR); ii) synthesized by, for example, chemical synthesis; iii) recombinantly produced by cloning; or iv) purified from a more complex molecule or from a mixture of molecules, such as by cleavage and size fractionation. Due to the degeneracy of the genetic code, many different oligonucleotide sequences can be identified which encode the extracellular domain of CD47 and which in particular, encode the CD47 epitope. Accordingly, various embodiments of the invention embrace the oligonucleotides which encode the CD47 extracellular domain (in particular, the CD47 epitope) but which have nucleotide sequences which differ from the sequences of the naturally-occurring CD47 gene or its allelic variants.

In addition to the foregoing oligonucleotides, the invention also provides an isolated "antisense" oligonucleotide that is capable of hybridizing under stringent conditions to the naturally-occurring CD47 nucleotide sequence to prevent transcription or translation. Preferably, the antisense oligonucleotide hybridizes to a nucleotide sequence located in the leader sequence of the CD47 cDNA (GenBank Accession No. S36644). Alternatively, the isolated oligonucleotide is capable of hybridizing under stringent conditions to a "unique fragment" (defined below) of the nucleotide sequence residing in the nucleic acid sequence which encodes the CD47 epitope. As used herein, the phrase "hybridizing under stringent

conditions" is a term of art which refers to the conditions of temperature and buffer concentration which will permit hybridization of a particular oligonucleotide or nucleic acid to its complementary sequence and not to non-complementary sequences. The exact conditions which constitute "stringent" conditions, depend upon the length of the nucleic acid sequence and the frequency of occurrence of subsets of that sequence within other non-identical sequences. By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, one of ordinary skill in the art can, without undue experimentation, determine conditions which will allow a given sequence to hybridize only with identical sequences. Suitable ranges of such stringency conditions are described in Krause, M.H. and S.A. Aaronson, Methods in Enzymology, 200:546-556 (1991). Stringent hybridization conditions, depending upon the length and commonality of a sequence, may include hybridization conditions of from 30 to 60°C and from 5x to 0.1x SSC. Highly stringent hybridization conditions may include hybridization at 45°C and 0.1 SSC. Less than stringent conditions are employed to isolate nucleic acid sequences which are substantially similar, allelic or homologous to any given sequence.

As used herein, the phrase "unique fragment" refers to a nucleic acid sequence having less than 25% sequence homology with previously identified nucleic acid sequences. More preferably, the unique fragments have less than 10% sequence homology with known nucleic acid sequences. Such unique fragments can be identified by searching the Genbank, PIR and/or Swiss-Prot data bases using conventional searching programs. The unique fragments are useful, for example, as probes and primers in nucleic acid hybridization assays and in amplification reactions, respectively.

For "antisense" applications, i.e., applications in which the isolated oligonucleotide is used to regulate transcription and/or translation of CD47, the preferred oligonucleotide is between about 10 and about 100 nucleotides in length. Preferably, the antisense oligonucleotide is capable of hybridizing under highly stringent conditions to unique fragments of the CD47 antigen. More particularly, "antisense oligonucleotide" refers to an oligonucleotide (DNA and/or RNA) that is capable of hybridizing to the naturally-occurring DNA or mRNA encoding the CD47 antigen. Base-pairing of the antisense oligonucleotide with the DNA (or RNA) encoding the CD47 antigen in vivo, prevents CD47-mediated neutrophil transmigration across a cell layer (e.g., the epithelium) by preventing transcription (or translation) of CD47 in vivo.

Methods for expressing the above-identified oligonucleotides in a suitable expression system including a host cell are well known to those of ordinary skill in the art (see, e.g., Sambrook, et al., Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Plainview, NY (1989)). The term "host cell" refers to a prokaryotic or eukaryotic cell which, together with a recombinant vector, comprises an expression system. The term host cell also embraces a host cell in which the vector or isolated oligonucleotide has integrated into the host cell nucleic acid. In a preferred embodiment, the expression vector includes at least one strand of the above-disclosed isolated oligonucleotide. Preferably, the oligonucleotide is operatively joined to at least one regulatory sequence, e.g., a promoter sequence, an enhancer sequence. A coding sequence (e.g., the isolated oligonucleotide) and a regulatory sequence are said to be operatively joined when they are linked in such a way as to place expression of the coding sequence under the influence or control of the regulatory sequence.

Suitable cell lines include mammalian cells (e.g., Chinese hamster ovary cells (CHO), monkey COS10C7 or 19 cell); bacterial cells (e.g., E. coli, B. subtilis and Pseudomonas strains); insect cells (e.g., SF9) and various yeast strains. Exemplary procedures for obtaining expression of a foreign gene in the above-identified cell lines are disclosed in U.S. 5,211,657, the entire contents of which are incorporated herein by reference.

Examples

The role played by CD47 in modulating PMN transmigration across the epithelium was investigated by: (1) developing anti-epithelial cell hybridomas; (2) screening the hybridomas for the ability to inhibit PMN transmigration across and epithelial cell monolayer in a transmigration assay; (3) subcloning a hybridoma of interest (the C5/D5 antibody) and using the antibody to immunoprecipitate an epithelial cell antigen; (4) biochemically characterizing the immunoprecipitated epithelial cell antigen as CD47; and (5) identifying the CD47 epitope to which the C5/D5 antibody binds. Biochemical analysis demonstrated that the C5/D5 antibody which inhibited PMN transmigration across an epithelial cell monolayer did not inhibit adhesion between the PMN and the cells of the monolayer. Each of the foregoing steps is described in detail in the Examples.

EXAMPLE 1 - **Preparation and Characterization of Antibody C5/D5 IgG**

A. Methods

(1) Cell Culture

T84 cells (Dharmsathaphorn, K. and J. Madara (1990) Methods Enzymol. 192:354-389; Dharmsathaphorn, K. et al. (1984) Am. J. Physiol. 246:6204-8) were grown in a 1:1 mixture of Dulbecco's modified Eagle medium and Hams F-12 medium supplemented with 15mM HEPES buffer (pH 7.5), 14 mM $NaHCO_3$, 40μg/ml penicillin, 8μg/ml ampicillin, 90μg/ml streptomycin and 5% newborn calf serum. Subculturing (or preparation of suspensions and / or lysates) was performed every 6-8 days by treatment with 0.1 % trypsin and 1.0 mM EDTA in Ca^{++} and Mg^{++} free phosphate buffered saline (Dharmsathaphorn, K., and J. Madara, L. (1990) Methods Enzymol. 192:354-389). For apical-to-basolateral transmigration experiments, T84 monolayers were grown on permeable collagen-coated, polycarbonate supports (inserts) with a surface area of 0.33 cm^2 (Costar Inc., Cambridge MA) as previously described (Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12). For physiologically-directed, basolateral-to-apical transmigration, T84 cells were plated on the underside of permeable filters to produce inverted monolayers (Madara, J.L. et al. (1992) J. Tiss. Cult. Meth. 14:209-216; Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12). Such inverted monolayers effectively reverse the polarity of neutrophil - epithelial interactions studied by allowing gravitational settling of PMN onto the basolateral aspect of the monolayer (Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12).

For purification of the protein recognized by mAb C5/D5 the clonal derivative, Cl.19A, of the human intestinal epithelial cell line HT29 (Augeron, C. and C. Laboisse (1984) Cancer Research 44:3961-3969) was grown to confluency in 165 cm^2 tissue culture flasks. Although the particular clonal derivative used for these experiments was provided by Dr. Christian Laboisse (Univerisite' de Nantes, Nantes, France) and is described in the Augeron and Laboisse reference, other starting materials from which the protein recognized by mAb C5/D5 could have been purified include, e.g., HT29 cells (available from the ATCC, Rockville, MD) and human spleen cells from patients diagnosed as having chronic myelogenous leukemia (CML).

Subculturing (or harvesting) of the cells was performed every 5 days by trypsin treatment with 0.1 % trypsin in Ca^{++} and Mg^{++} free phosphate buffered saline. Typically, cells were split 1:10 in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% FBS, 1mM L-glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin (all from Gibco BRL, Grand Island, NY) and became confluent within 5-6 days.

Primary cultures of human umbilical vein endothelial cells (HUVEC) were established from normal term umbilical cords as described previously (Gimbrone, M.A. (1976) Progress in hemostasis and thrombosis, T. Spaet, editor. Grune & Stratton Inc., New York, 1-28). For

experimental use, second passage cells were plated on gelatin-coated 0.33cm² polycarbonate filters (Costar Corp., Cambridge, MA) and maintained in Medium 199 (with 25mM HEPES, Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 100U/ml streptomycin, 25μg/ml endothelial cell growth supplement (Collaborative Research Inc., Bedford, MA), 50μg/ml heparin (Sigma, St. Louis, MO), and 250ng/ml amphotericin B (Fungizone, Gibco BRL) for 7 days prior to use.

For surface labeling experiments by ELISA, T84 or HT29 (Cl 19.A) cells were plated 48 hours prior to use at 3/4 confluent density in 96-well microtiter plates in cell culture media with or without 1000U/ml IFNγ (Genentech Inc., South San Francisco,CA).

(2) PMN Isolation

PMN were isolated from whole blood (anticoagulated with citrate/dextrose) obtained from normal human volunteers, using a gelatin sedimentation technique previously described in detail (Henson, P. and Z.G. Oades (1975) J. Clin. Invest. 56:1053-61). PMN were resuspended in modified HBSS devoid of Ca^{++} and Mg^{++} (HBSS(-)) at a concentration of $4x10^7$ cells/ml (4°C) and used for subsequent experiments.

(3) Buffers

HBSS consisted of (in g/L); $CaCl_2$ 0.185, $MgSO_4$ 0.098, KCl 0.4, KH_2PO_4 0.06, NaCl 8, Na_2HPO_4 0.048, glucose 1, and HEPES added to 10 mM (pH 7.4). HBSS(-) was prepared as HBSS but without $CaCl_2$ or $MgSO_4$. Blocking buffer consisted of a phosphate buffered saline containing 2mM $MgCl_2$, 1mM $CaCl_2$, 10mM dextrose and 0.5% heat treated BSA (heated to 60°C). IPPT wash buffer consisted of 400mM NaCl, 100mM NaF, 1mM EDTA, 1% Triton X-100 and 10mM $NaHPO_4$ pH 7.4. Lysis buffer was prepared as a solution of 100mM KCl, 30mM NaCl, 2mM EDTA, 10mM HEPES pH 7.4, and 2% Triton X-100. Sample buffer consisted of 2.5% SDS, 0.375M Tris pH 6.8, 20% glycerol, and 0.1% bromphenol blue.

(4) Miscellaneous Biochemical Assays

Protein was assayed using the Bradford method (Bradford, M. (1976) Anal. Biochem. 72:248-254), and by the BCA method as described by Pierce Inc. using bovine γ globulin as a standard. Superoxide production was measured as the superoxide dismutase inhibitable reduction of cytochrome C as previously described (Parkos, C.A. et al. (1985) J. Biol. Chem. 260:6541-7). Lactoferrin release was quantitated by ELISA as previously described (Parkos, C.A. et al. (1985) J. Biol. Chem. 260:6541-7).

(5) Membrane Preparation

T84 epithelial membranes for immunization were prepared as described previously (Kaoutzani, P. et al. (1993) Am.J.Physiol.264 (Cell Physiol.) 33:C1327-C1335). Briefly, T84 cells, plated as monolayers on 45 cm^2 permeable supports (rings) (Costar Inc.) or on 150 cm^2 tissue culture flasks, were cooled to 4°C, washed with Hanks balanced salt solution (HBSS), and cells were scraped from the support with a teflon spatula in a small volume of homogenization buffer consisting of 0.34M sucrose, 10mM HEPES pH 7.3, 1mM ATP, 1mM Dithiothreitol and 0.1mM EDTA. Scraped cells are then treated with 2.5mM diisopropylfluorophosphate (DFP) (15 min, 4°C) followed by nitrogen cavitation (200 psi, 8 minutes, 4°C). The cavitate was centrifuged at 1000xg to remove nuclear debris and the NaCl content of the supernatant adjusted to 1.0M to remove peripheral membrane proteins. The resulting membrane suspension was pelleted by ultracentrifugation at 100,000xg for 45 minutes and was resuspended in homogenization buffer at an equivalent cell density of 1-2x10^8 per ml and stored at -80°C until further use.

(6) Antibodies

To identify ligands important in neutrophil - epithelial interactions, monoclonal antibodies were prepared against T84 cell membranes and screened for inhibition of neutrophil - T84 interactions. Female BALBc mice were immunized by intraperitoneal injection of T84 epithelial membranes (200µl per mouse; representing 1x10^7 cell equivalents emulsified with an equal volume of complete Freunds adjuvant). Two subsequent intraperitoneal immunizations were performed over the next six weeks with the same material emulsified with incomplete adjuvant. Mice with high anti-epithelial antibody titers were given a final intravenous immunization by tail vein (50µl T84 membranes in HBSS) and the spleens were harvested for fusion 4 days later. Splenocytes were fused with P3U1 myeloma cells using 1500 MW polyethylene glycol (Boehringer Mannheim, Germany) and resuspended in standard selection media (RPMI supplemented with 1mM L-glutamine, 1/100 dilution of non-essential amino acids, 100U/ml penicillin, 0.1 mg/ml streptomycin (all from Gibco BRL, Grand Island, NY), 1mM sodium pyruvate, 10% heat inactivated FBS, and HAT (1/1000 dilution of a stock of hypoxanthine, aminopterin and thymidine; American Type Tissue Collection)). The resultant hybridomas were plated at limiting dilution and cultured in 96-well tissue culture plates in the presence of thymocytes prepared from DBA2 mice at a density of 1.2x10^5 splenocytes and 5x10^5 thymocytes per well. After ~7-10 days of growth, the supernatants from wells containing ~1mm sized colonies were harvested and assayed for surface reactivity with both PMN and T84 monolayers

by ELISA as described below. Wells demonstrating predominantly epithelial reactivity were transferred to 24-well tissue culture plates for expansion and production of cell culture supernatant. The tissue culture supernatants were then removed and frozen in aliquots for subsequent screening in the transmigration and adhesion assays described below. Hybridomas from the 24-well culture plates were frozen and stored until screening by functional assay was complete. After identification of functionally inhibitory hybridoma supernatants, the corresponding hybridomas were thawed, subcloned by limiting dilution x3 and weaned from selection media. Antibodies were isotyped using a Dipstick Isotype Kit according to the manufacturers instructions (Gibco, BRL) and hybridoma cells were injected into the peritoneal cavities of pristane-primed mice (2-5x10^6 cells per mouse) for the production of ascites fluids.

Antibodies were purified from ascitic fluid by standard procedures using protein-A sepharose (Sigma, St. Louis, MO) followed by dialysis against 150mM NaCl containing 10mM HEPES pH 7.4. Aliquots of concentrated, purified antibody (1.5 - 3mg/ml) were frozen for use in functional assays. $F(ab')_2$ and Fab' preparations were obtained by pepsin digestion (100U/mg, 6h, 37°C) followed by cysteine reduction (10mM, 2h, 37°C) and alkylation as described in detail elsewhere (Parham, P. (1983) in Immunological Methods in Biomedical Sciences, D. M. Weir, et al. eds., Blackwell, Oxford, 14.1-14.2). Purity of antibody digests was confirmed by SDS-PAGE under reducing and non-reducing conditions.

Other commercially available antibodies were used as controls. As a positive control for inhibition of neutrophil transmigration, antibody 44a (anti-CD11b; American Type Tissue Collection) was used as described previously (Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12). Another anti-CD11b mAb which is non-inhibitory but used for immunofluorescence was OKM1 (Wright, S. D. et al. (1983) Proc. Natl. Acad. Sci. 80:5699-703)(American Type Tissue Collection). Antibody W6/32 (antibody to major histocompatibility antigen class 1) served as a binding, non-inhibitory control (Barnstable, C.J. et al. (1978) Cell 14:9-20). Anti-CD47 (mAb BRIC 126 (Avent, N.P. et al. (1988) Biochem J. 251:499-505; Mawby, W.J. et al. (1994) Biochem J. 304:525-30) was obtained from Biosource Intl., Camarillo, CA.

(7) Antibody Labelling/Immunoprecipitation

ELISA for detecting cell surface binding antibodies. Confluent T84 monolayers in 96-well plates (~2x10^5 cells per well) were treated with 2mM EDTA (4 min, 37°C) to expose basolateral epitopes (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479), cooled to 4°C and incubated for 2h with 25µl of hybridoma supernatant or antibody solution. For neutrophil

surface binding, $2x10^5$ PMN in HBSS were placed in each well and allowed to attach and spread for 30 min (37°C) followed by cooling to 4°C and blocking non-specific binding with cell culture media containing 10% FBS. Antibody solutions were then added as outlined above. After subsequent gentle washing with HBSS, cells were incubated with 25µl of enzyme-conjugated secondary antibody diluted 1:1000 in HBSS/10% goat serum (1h, 4°C). Secondary enzyme conjugates included peroxidase for T84 cells and alkaline phosphatase for PMN. Color was developed using standard substrate assays and the plates were read in a microtiter plate reader. In some experiments, FITC-conjugated goat anti-mouse secondary antibody was used. ELISA assays performed with such fluorescent secondary antibody were quantitated using a fluorescence microtiter plate reader (Millipore Inc., Milford, MA).

Immunofluorescence. For immunofluorescence, T84 monolayers were fixed in 3.7% paraformaldehyde in HBSS (10 minutes, 20°C), washed and incubated in HBSS containing 5% normal goat serum (NGS) for 30 minutes followed by primary antibody for 2h (10µg/ml in 5% NGS). After washing, monolayers were incubated with FITC-conjugated 2° antibody (Cappel Inc., Durham, NC)(1 hour, 20°C) and mounted in PBS-glycerol-p-phenylene-diamine. Labelled monolayers were then viewed with a Zeiss/BioRad MRC-600 confocal fluorescence microscope. As a control for background labeling, control monolayers were incubated with comparable concentrations of normal mouse IgG and secondary antibody. Labelling was also performed on 3µ frozen tissue sections of human colonic mucosa obtained from fresh surgical specimens. Tissue sections, mounted on glass coverslips, were air-dried and followed by fixation in 3.7% paraformaldehyde and fluorescently labelled as above.

Flow cytometry. PMN were analyzed for surface expression of CD47 by flow cytometry as previously described (Colgan, S.P. et al. (1995) J. Biol. Chem. 270:10531-10539) using a FACScan flow cytometer (Becton-Dickson Immunocytometry Systems, Mountain View, CA).

Immunoprecipitation experiments. To identify protein antigens of functionally inhibitory antibodies, immunoprecipitation experiments were performed, after cell surface labelling with biotin, on T84 and HT29 monolayers cultured on either $5cm^2$ permeable supports or on plastic. Briefly, monolayers were washed with HBSS and labelled with a solution of 1mM sulfo-NHS biotin (Pierce, Rockford, IL) in HBSS for 20 minutes (4°C) followed by quenching the reaction with 150mM NH_4Cl. Each $5cm^2$ monolayer was solubilized in 1ml of lysis buffer containing 100mM KCl, 30mM NaCl, 2mM EDTA, 10mM HEPES pH 7.4, 2% Triton X-100 and protease inhibitors including 1.25mM PMSF, 5µg/ml chymostatin, 1µg/ml each of leupeptin, pepstatin

and bestatin (4°C). For cells grown in flasks, lysis buffer was added at a ratio of roughly 1 ml per 75 cm^2. The T84 cell lysate was subjected to sequential low speed (3000xg, 10 min) and high speed (180,000xg, 45 min) centrifugation followed by filtration (0.2μ filter). The filtered lysate was precleared for 2h with 50μl of IgG-sepharose (mouse IgG coupled to CNBR activated sepharose 6MB at a protein/sepharose density of 3mg/ml according to the manufacturers instructions (Pharmacia Inc, Upsala, Sweden)) followed by incubation for 2h (4°C) with 30μl C5/D5 - sepharose, prepared exactly as described for the mouse IgG-sepharose above. Immunoprecipitates were washed first in IPPT wash buffer followed by 1% octylglucoside in 100mM sodium phosphate pH7.4 and finally washed in 1% octylglucoside in 20mM sodium phosphate pH 7.4. The washed immunoprecipitates were denatured by heating to 100°C in the presence of 50μl non-reduced sample buffer followed by removal of the sepharose pellet. The denatured, solubilized immunoprecipitate was then subjected to reduced and non-reduced SDS-PAGE on linear 4-16% gradient polyacrylamide gels followed by western blotting using standard protocols. Prior to SDS-PAGE, reduced samples (dithiothreitol added to 20mM; 100°C, 3 min) were alkylated by addition of iodoacetamide to 50mM (100°C, 3 min) and non-reduced samples were alkylated by addition of iodoacetamide to 5mM. Biotin surface-labelled proteins were visualized after incubation with peroxidase conjugated streptavidin using enhanced chemiluminescence, according to the manufacturers instructions (Amersham Inc. Buckinghamshire, UK).

For deglycosylation experiments, immunoprecipitates were denatured in buffer containing 0.5% SDS followed by addition of a 7-fold excess of NP-40. Samples were then subjected to N-linked or O-linked deglycosylation using commercially available enzymes (Peptide: N-glycosidase F, neuraminidase and O-glycopeptide endo-D-galactosyl-N-acetyl-α-galactosamino hydrolase respectively) exactly as described by the manufacturer (N-glycanase, neuraminidase, O-glycanase; Genzyme, Cambridge, MA).

(8) Protein Purification and Microsequencing

Functionally active CD11b/CD18 was purified by immunoaffinity chromatography using peripheral blood leukocyte lysates exactly as previously described (Diamond, M.S. et al. (1990) J. Cell Biol. 111:3129-39).

Immunopurification of the antigen defined by C5/D5 IgG. Approximately 8,500 cm^2 of Cl 19.A HT29 cells were stimulated with 1000U/ml IFNγ for 48h in order to increase the expression of the C5/D5 antigen. Immediately before harvesting, five 165cm^2 flasks (~10% of

the total) were transiently exposed to 2mM EDTA in HBSS(-) to open tight junctions thereby exposing ectodomains of basolateral membrane proteins (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479). The EDTA-treated cells were then surface labelled with biotin as described above. Both labelled and unlabeled flasks were then washed 3x with HBSS (4°C), and cells were isolated and pooled by scraping with a teflon spatula into ~120ml of lysis buffer (2-3ml of lysis buffer per flask of cells) containing 1.25mM PMSF, 5μg/ml chymostatin, 1μg/ml each of leupeptin, pepstatin and bestatin (4°C) and 2mM EDTA. Diisopropylfluorophosphate (Sigma, St. Louis, MO) was then added to the lysate to achieve a final concentration of 2.5mM and stirred for 15 minutes on ice. The extract was sequentially subjected to low speed (2000xg, 10 min) then high speed (180,000xg, 45 min, 4°C) centrifugation followed by passage through a 0.2μ filter. The extract was then pumped at a flow rate of 25ml/h first through a column of bovine γ globulin-sepharose (BGG-sepharose, Sigma, St. Louis, MO) (5ml, 3mg γ globulin per ml of beads; coupled as described in the above immunoprecipitation section) followed in tandem by a column of C5/D5-sepharose (3ml, 3mg IgG per ml of beads; coupled as described above). The C5/D5 column was then washed at a flow rate of 25ml/h with IPPT wash buffer (50ml) followed by 1% octylglucoside in 100mM sodium phosphate pH7.4 (30ml) and finally in 1% octylglucoside in 20mM sodium phosphate pH 7.4 (30ml). Bound proteins were eluted at a flow rate of 25ml/h with a 30ml pH gradient decreasing from pH 5.0 (150mM NaCl and 50mM NaOAC, 1% n-octylglucoside) to pH 3.0 (150mM NaCl and 100mM glycine/HCl, 1% n-octylglucoside) followed by an additional 10ml of pH 3.0 elution buffer. Fractions of 2ml were collected and neutralized by the addition of 0.1ml of 2.0M Tris pH 8.0 and were analyzed by SDS-PAGE and western blotting as described above.

For protein microsequence, the peak fraction of immunopurified protein was concentrated ~200 fold (Centricon 30 microconcentrator; Amicon Inc, Beverly, MA) and subsequently denatured, reduced and alkylated by the sequential addition of sample buffer containing 20mM dithiothreitol followed by iodoacetamide to 50mM. The sample was subjected to SDS-PAGE as a single lane on a 4-16% gradient polyacrylamide gel followed by electrophoretic transfer to polyvinylidene difluoride membrane (Immobilon-P; Millipore Inc., Bedford, MA). The transferred protein was visualized by stain with amido black followed by excision of the band (approximately 50mm^2) and submission to the Harvard Microchemistry Service (Cambridge, MA) for tryptic digest and internal microsequencing as previously described (Aebersold, R.H. et

al. 1987) Proc. Natl. Acad. Sci. 84:6940-6974; Lane, W. et al. (1991) J. Prot. Chem. 10:151-160).

(9) Transmigration Experiments

PMN transmigration experiments were performed using both standard (apical-to-basolateral migration) and inverted (basolateral-to-apical migration) T84 monolayers cultured on 0.33cm^2 permeable supports as previously described (Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12). Briefly, confluent T84 monolayers were washed free of media followed by apical or basolateral addition of 50µl of antibody solution in HBSS and incubation for 20 minutes (20°C). For some apical-to-basolateral transmigration experiments T84 monolayers were pre-exposed to 2mM EDTA in HBSS(-) for 12 minutes prior to washing with HBSS. Such transient calcium chelation has been shown to expose basolateral ligands to the apical compartment of the transwell device without grossly altering the morphology of the epithelium (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479). After a 20 minute preincubation, HBSS was added (100µl) followed by 1x10^6 PMN in 25µl HBSS(-). Transmigration was initiated by transfer of antibody/PMN containing monolayers to 24 well tissue culture plates containing 1ml of 1µM fMLP in HBSS. After incubation for 110 minutes at 37°C, neutrophil migration across monolayers into the chemoattractant - containing lower chambers was quantitated by myeloperoxidase assay (Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12).

In experiments examining the effect of cytokine preactivation on PMN transmigration, the cell culture media on confluent T84 monolayers was replaced with media containing maximally stimulating concentrations of IFNγ (1000U/ml) or IL-4 (10U/ml) followed by culture for 48h as previously described (Colgan, S.P. et al. (1993) J. Cell. Biol. 120:785-798; Colgan, S.P. et al. (1994) J. Immunol. 153:2122-2129). Cytokine - activated monolayers were then washed in HBSS and used in transmigration assays.

PMN transendothelial migration using monolayers of human umbilical vein endothelial cells (HUVECs) was assessed in a fashion exactly as described above for T84 monolayers except for the use of a 10nM transendothelial gradient of fMLP.

In subsets of experiments, monolayers or PMN were pre-treated with antibody followed by antibody washout and use in subsequent transmigration assays. In such experiments, monolayers (20°C) or PMN (2x10^6 cells/ml in HBSS(-), 4°C) were preincubated with antibody in HBSS for 30 minutes followed by extensive washing and subsequent transfer to transmigration assays as described above. For monolayer pre-incubation experiments, unbound antibody was washed out by five successive rinses in HBSS with a five minute incubation in 1ml

of HBSS after each rinse. In such experiments, each rinse was effective in reducing the unbound antibody concentration by greater than one order of magnitude thereby reducing the final concentration of unbound antibody to negligible values. Other transmigration experiments were performed on collagen coated permeable supports without any epithelial cells. In such assays, collagen coated inserts were incubated overnight in sterile tissue culture media followed by washing and placement into 24-well tissue culture plates containing 1ml of antibody in HBSS. After addition of 0.15ml of antibody solution and $1x10^6$ PMN to the upper chamber, fMLP was added to the lower chamber to a final concentration of 10nM. PMN transmigration was then assessed exactly as above.

(10) Adhesion Experiments

The effects of C5/D5 IgG on neutrophil adhesion to T84 monolayers was studied using previously described methods (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479). Briefly, confluent T84 monolayers on permeable supports were transiently pre-exposed to 2mM EDTA in HBSS(-) for 12 minutes followed by washing in HBSS. To the apical surface of each monolayer, 50µl of antibody solution in HBSS containing 100nM fMLP was added followed by transfer of the monolayers to 24-well tissue culture plates containing 1ml of HBSS per chamber. $2x10^6$ PMN were added in 50µl followed by centrifugation at 250xg for 4 minutes (20°C). Transwells were then allowed to incubate at 37°C for 10 minutes followed by washing and quantitation of adherent PMN by myeloperoxidase assay.

The effects of C5/D5 IgG on T84 cell binding to CD11b/CD18 was assayed using slightly modified, previously described methods (Diamond, M.S. et al. (1990) J. Cell Biol. 111:3129-39). Microtiter plates were coated with functionally active CD11b/CD18 which was purified as described above. For optimal coating with CD11b/CD18, a solution of purified integrin at ≥0.1mg/ml was diluted 15-fold with 150mM NaCl, 2mM $MgCl_2$, 25mM Tris pH7.3 and allowed to bind to microtiter wells for 2h (20°C). As described previously (Diamond, M.S. et al. (1990) J. Cell Biol. 111:3129-39), nonspecific binding was blocked by incubation with a solution of blocking buffer containing 0.5% heat - treated bovine serum albumin. For cell binding assays, trypsin / EDTA elicited T84 cells were fluorescently labelled for 10 minutes at 37°C by incubation with 5µg/ml BCECF-AM (2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; Molecular Probes, Inc. Eugene, OR.).

Adhesion assays were then performed by the addition of 50µl of antibody solution in blocking buffer to the CD11b/CD18 coated microtiter plates followed by a 20 minute incubation

(20°C). Labelled epithelial cells (50µl, ~ $2.5x10^5$ cells per well) were added followed by gentle, constant swirling for 15 minutes to allow antibody binding but prevent adhesion (20°C). The assay was then moved to a flat, stationary surface at 37°C for 1h to allow for adhesion. To quantitate adhesion, each well was gently washed twice and total fluorescence of each well was assayed at an excitation/emission wavelength of 485/535 nm using a fluorescence microtiter plate reader (Millipore Inc, Milford, MA). In such assays, the percentage of applied cells adherent to purified CD11b/CD18 typically ranged from 25 to 55%. Percent adherence was calculated as the fluorescence ratio (post-wash fluorescence / pre-wash fluorescence) x 100.

(11) Statistical Analysis

Data are presented as the mean ± SD and compared by Students t-test or by one-way analysis of variance (ANOVA).

B. Results

Three fusions of splenocytes from mice immunized with T84 membranes yielded approximately 4,300 antibody producing clones which were screened for reactivity with both neutrophils and T84 epithelial cells. Using the differential screening approach outlined above, approximately 350 clones were identified which reacted primarily with the external surface of epithelial cells. Of several antibodies which were subcloned, one antibody - C5/D5 (IgG1), which was among those which inhibited neutrophil-epithelial interactions, was further characterized.

(1) C5/D5 IgG Inhibits PMN Transepithelial Migration But Not Adhesion To Purified CD11b/CD18:

The effects of C5/D5 IgG on neutrophil migration across T84 monolayers in the apical-to-basolateral or basolateral-to-apical direction are depicted in Figure 1. As shown in Figure 1A, PMN transepithelial migration in the apical-to-basolateral direction is markedly inhibited by C5/D5 IgG at test sample concentrations as low as 3µg/ml ($4.5\pm3.9x10^4$ vs 20.5±0.45 for C5/D5 vs binding control (W6/32); $p<.005$). The actual inhibitory concentration of antibody used in the assay is one-fourth of the antibody concentration in the test sample since one part of the test sample is diluted with three parts buffer in the assay. Thus, Figure 1A shows that PMN transmigration is inhibited about 75% by C5/D5 IgG at concentrations in the assay as low as 0.75 µg/ml. The effects of C5/D5 IgG on PMN migration in basolateral-to-apical directed transmigration (physiologically directed transmigration) are shown in Figure 1B. Again, PMN

transmigration was markedly inhibited by C5/D5 IgG in the range of 3 to 12 μmg/ml (test sample concentration) (11±4.1 vs 36.5±8.5x10^4 for 12.5 μg/ml C5/D5 vs ctl (W6/32); p<.02).

Since the C5/D5 epitope appeared crucial to PMN transepithelial migration and we have previously shown that transepithelial migration is modulated by cytokines such as IL-4 and IFNγ (Colgan, S.P. et al. (1993) J. Cell. Biol. 120:785-798; Colgan, S.P. et al. (1994) J. Immunol. 153:2122-2129), we next determined whether the C5/D5 epitope was also functionally involved in transmigration following epithelial exposure to cytokines. As shown in Figure 1C, PMN transmigration across IL-4 pre-stimulated (10U/ml, 48 hr) T84 monolayers was also strongly inhibited by C5/D5 IgG at test sample concentrations as low as 2 μg/ml. As shown in Figures 1D and 1E, pre-treatment of T84 monolayers with IFN-γ (1000U/ml, 48 hr), which has been shown to influence rates of PMN transmigration and regulate surface expression of as yet undefined PMN ligands (Colgan, S.P. et al. (1993) J. Cell. Biol. 120:785-798), retains sensitivity to inhibition of PMN transmigration by C5/D5 IgG (Figure 1D) and results in enhanced surface expression of the C5/D5 epitope (Figure 1E; 250±35 vs 760±90 fluorescence units before and after IFN-γ stimulation respectively; p< .02). The data in Figure 1D shows mAb C5/D5 - inhibits PMN migration across IFN-g pretreated T84 monolayers irrespective of the polarity of transmigration (13.7±6.3 vs 35.6±4.7 x10^4 basolaterally-to-apically migrated PMN for C5/D5 IgG vs control respectively; p<.02). Of interest, such inhibition was observed despite IFN-γ-induced downregulation of physiologically-directed transmigration (Colgan, S.P. et al. (1993) J. Cell. Biol. 120:785-798) from 63.5±4x10^4 migrated PMN in controls to 35.6±4.7x10^4 migrated PMN after IFN-γ pretreatment. As revealed in cross-comparisons of the C5/D5 IgG concentration dependence between Figures 1A, 1C, and 1D, inhibition of apical-to-basolateral directed transmigration by this antibody was comparable between control and cytokine stimulated monolayers.

Next, assays of PMN adhesion to epithelia were performed in order to assess whether C5/D5 inhibited an initial adhesive event as opposed to a distal transmigration event. For these studies (Figure 2A), a previously detailed assay which permits exposure of PMN to both apical and basolateral epithelial ligands (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479) was utilized. Saturating concentrations of C5/D5 IgG did not inhibit adhesion of PMN to T84 monolayers thus resulting in PMN adherence values comparable to those obtained in the presence of a control binding antibody (W6/32) or in the absence of antibody (10.9±2.8 vs 9.6±2.8 and 10.3±0.9x10^4 adherent PMN for C5/D5 vs W6/32 CTL and no antibody

respectively; NS). Antibody 44a, previously shown to effectively interfere with initial PMN-epithelial adhesion by blocking PMN CD11b/CD18, served as a positive control for these experiments and inhibited PMN - epithelial adhesion by greater than 70%.

Given the dependence of PMN transepithelial migration on CD11b/CD18, we next determined whether C5/D5 IgG inhibited some form of epithelial adhesive engagement of this PMN integrin. Figure 2B shows the effects of C5/D5 IgG on T84 cell adhesion to purified, functionally active CD11b/CD18. In such assays, T84 cells strongly adhere to purified CD11b/CD18 in a specific manner. As shown in Figure 2B, 56±6.9% of the applied T84 cells adhere to CD11b/CD18 in the presence of a binding, control antibody, and such adherence is markedly inhibited (to 2.4±0.4 %) after treatment with blocking anti-CD11b antibody, 44a. In contrast to antibody 44a, pre-incubation of epithelial cells with saturating concentrations of C5/D5 did not influence the efficiency of epithelial cell adhesion to immobilized CD11b/CD18 (57±4 % of T84 cells adherent, NS). The lack of inhibition of C5/D5 IgG on T84 adhesion was not due to proteolysis of relevant epitopes by the trypsin elicitation procedure since the antibody was able to effectively surface label trypsinized T84 cells (0.579±0.02 versus 0.191±0.014 OD units for T84 cells labelled with C5/D5 IgG versus control mouse IgG1). As an additional test of the specificity of epithelial cell adhesion to purified CD11b/CD18, parallel assays were performed in microtiter wells coated with BSA only and showed no significant adhesion.

The inhibition of transmigration but not adhesion of PMN by mAb C5/D5 suggested the possibility of CD11b/CD18 independent, post-adhesive interactions between epithelia and PMN. If transmigration was inhibited at point(s) distal to initial adhesive events, one might expect to observe an accumulation of PMN in monolayers during transmigration assays performed in the presence of mAb C5/D5 (25ug/ml). Indeed, a frequent observation in our transmigration assays was a C5/D5 dependent increase in monolayer associated PMN. While transmigration into the opposite chamber was inhibited by 68±11%, there was nearly a doubling of monolayer - associated PMN after incubation with C5/D5 IgG. A confocal fluorescence micrograph of a T84 monolayer after transmigration in the presence of C5/D5 IgG demonstrated that the quantitative increase in monolayer-associated PMN correlated with the accumulation of PMN within the epithelial monolayer. These results suggest that mAb C5/D5 IgG acts by inhibiting PMN movement across the epithelium by influencing events subsequent to initial CD11b-dependent adhesive events.

(2) The Antigen Defined by C5/D5 is an ~ 60kDa Membrane Glycoprotein

Experiments were next performed to identify the antigen recognized by C5/D5 IgG. Since C5/D5 IgG recognized an extracellular ligand, polarized monolayers on permeable supports were surface labelled (apical and basolateral) with biotin, detergent solubilized and immunoprecipitated with immobilized C5/D5 IgG. Samples were then subjected to SDS-PAGE on 4-16% gradient polyacrylamide gels followed by western blot, incubation with streptavidin-peroxidase and development by enhanced chemiluminescence. C5/D5 IgG specifically immunoprecipitated a membrane protein appearing as a broad band centered at ~60kD under reducing conditions and with a similar, perhaps slightly larger (~60-65kD), apparent molecular mass under non-reducing conditions. Deglycosylation experiments revealed that removal of N-linked sugar residues with treatment by peptide:N glycosidase F caused a marked reduction in the apparent molecular mass to ~35kD. When this deglycosylated immunoprecipitate was subsequently subjected to conditions which remove O-linked sugars (O.glycanase) no further reduction in molecular mass was apparent. Although reduced and alkylated, the immunopurified protein occasionally exhibited a "laddering effect", presumably due to oligomerization which resulted in the appearance of a more lightly labelled band of Mr ~100 kD. Thus, the antigen defined by mAb C5/D5 is a membrane protein with an apparent molecular mass of ~60kD, is heavily glycosylated with N-linked carbohydrate and has a core polypeptide molecular mass of ~35kD.

(3) Purification, microsequence and identification of the C5/D5 antigen as CD47

HT 29 cells (subclone Cl 19.A) were used to bulk purify the antigen recognized by C5/D5. Cl 19.A cells are a well differentiated human intestinal epithelial cell line with growth characteristics more logistically suited for large-scale tissue culture compared to the relatively slow-growing T84 cells. Surface expression of mAb C5/D5 epitope was assayed on control ((-)IFNγ) and IFNγ stimulated ((+) IFNγ) Cl19.A HT29 cells by ELISA as described above. On the Y axis, surface label was represented by optical density units after substrate addition. Specific labeling was determined by subtracting the optical density of monolayers incubated in normal mouse IgG. W6/32 IgG, which strongly labels epithelial cells, was used as a binding control antibody at 20μg/ml. The surface expression of the C5/D5 antigen, assessed by ELISA, on Cl 19.A HT29 cells, as with T84 cells, was shown to be responsive to IFNγ (48h, 1000U/ml). An approximately two-fold increase in surface expression of the C5/D5 antigen was induced by this cytokine and paralleled an increase in MHC class I expression. Moreover, C5/D5 immunoprecipitates obtained from Cl 19.A HT29 cells, like those obtained from T84 cells,

revealed a broad ~60kD band. While IFN-γ stimulation increased the yield of the antigen, there was no effect on the Mr of the immunoprecipitate.

Approximately 8,500 cm^2 of IFN-γ activated Cl 19.A HT29 cells were used to obtain sufficient quantities of the C5/D5 antigen for microsequence. The crude detergent extract was then sequentially passed through 5 and 3ml columns of bovine γ globulin-sepharose and C5/D5 IgG-sepharose, respectively. Fractions of 2ml were collected and neutralized as described above. The C5/D5 IgG column then was washed in a high salt buffer containing Triton X-100, followed by washing in buffers containing 1% n-octylglucoside and decreasing amounts of sodium phosphate (pH 7.3). The bound antigen then was eluted with a gradient of decreasing pH from 5.0 to 3.0 in buffer containing 1% n-octylglucoside. From 219mg of crude Cl 19.A HT29 cell lysate, approximately 50-75μg of purified protein was obtained, representing an overall purification of ~3000 fold. The avidin blot (a western blot of the column eluate fractions developed by enhanced chemiluminescence after incubation with streptavidin-peroxidase) and silver stain (the silver stained SDS gel of the column eluate fractions) of the purified material eluted from the C5/D5 - sepharose using a decreasing pH gradient showed a single biotin labelled protein band which was indistinguishable from the immunoprecipitate. Silver stain of the corresponding unconcentrated fractions confirmed purification to apparent homogeneity revealing a single protein band with a reduced apparent molecular mass of ~60kD.

The peak protein containing fraction was concentrated, subjected to SDS-PAGE and electrophoretically transferred onto a PVDF membrane followed by protein staining with amido black. Limited amino acid composition revealed ~124 pmol of protein immobilized on the PVDF membrane. Two different tryptic peptides were isolated by HPLC and sequenced yielding the following sequences: IEVSQLLK (SEQ. I.D. No. 34) and STVPTDF(S)(S)A (SEQ. I.D. No. 35), respectively (where parentheses indicate residues determined with lower confidence). Searches for sequence homology using GenBank/EMBL revealed a complete match for both peptides with a membrane protein referred to as OVTL3 (Campbell, I.G. et al. (1992) Cancer Res. 52:5416-20) or Integrin Associated Protein (IAP) (Lindberg, F.P. et al. (1993) J Cell Biol. 123:485-96), previously determined to be identical to CD47 (Lindberg, F.P. et al. (1994) J Biol Chem. 269:1567-70; Mawby, W.J. et al. (1994) Biochem J. 304:525-30). In addition to providing 100% identity with CD47, the GenBank search yielded no other significant sequence homology. Figure 3 shows the alignment of the two peptide sequences we obtained with that of CD47. In the lower half of Figure 3 is a hypothetical secondary structural model first proposed

by Lindberg et al (Lindberg, F.P. et al. (1993) J Cell Biol. 123:485-96) with the location of the two peptide sequences shown.

To confirm the homology between the antigens defined by C5/D5 and CD47, an ELISA and western blots were performed. Using microtiter wells coated with immunopurified C5/D5 antigen, the binding of C5/D5 IgG and commercially available anti CD47 antibody (BRIC 126) were compared. A standard ELISA was performed on microtiter wells coated with the purified material which had been diluted 15-fold with PBS and allowed to bind non-specifically to the surface (2h, 20°C). Primary antibodies included C5/D5 IgG as a positive control (1μg/ml) and a 1:10 dilution of commercially available anti-human CD47 (BRIC 126). Optical density (OD) was determined after substrate addition to alkaline phosphatase-conjugated secondary antibody. Non-specific background was determined using CTL IgG, i.e., by incubation with an irrelevant mouse IgG. Both C5/D5 IgG and anti-CD47 reacted strongly with the immunopurified material. Such cross-reactivity was also confirmed by western blotting of the commercially available anti-CD47 antibody against the immunopurified C5/D5 antigen.

The identification of the C5/D5 antigen as CD47 was surprising given its broad tissue distribution and the fact that hybridomas were initially screened for preferential binding to epithelial cells over PMN. The initial screening assays for the C5/D5 hybridoma suggested a low amount of surface labelling of T84 cells (approximately 0.1 OD unit above background) and even lower amounts on PMN. Flow cytometry of purified, non-permeabilized human neutrophils using C5/D5 IgG revealed substantial surface labelling with mean channel fluorescence values of 1 and 257 for control versus C5/D5 labelled PMN respectively. PMN were stained using mAb C5/D5 or no primary Ab (CTL) followed by FITC-labelled goat anti-mouse antibody. Histograms representing specifically stained cell numbers on the vertical axis (labelled counts) were plotted against fluorescence on a log scale from 20,000 cells per condition. Immunopurification of the C5/D5 antigen from 10g of PMN revealed a broadly staining protein band of ~60kD which was indistinguishable from that obtained from epithelial cells.

(4) Localization of C5/D5 Antigen in Intestinal Epithelia and Mucosa

The results of immunostaining experiments, performed with C5/D5 IgG, on polarized T84 cells and on frozen sections of human colonic mucosa are described herein. Paraformaldehyde fixed T84 monolayers were incubated with C5/D5 IgG for 1h (10μg/ml) followed by labelling with FITC conjugated secondary antibody as described in the methods. Mounted, stained

monolayers were then visualized by fluorescence confocal microscopy. The control was stained with normal mouse IgG. An X-Y fluorescence image in the mid-zone (subjunctional) of a T84 monolayer stained with C5/D5 showed a "chicken wire" or basolateral membrane staining pattern.

Paraformaldehyde fixed 3μ frozen sections of human colon were labelled with C5/D5 IgG followed by FITC-conjugated secondary as described above and in the methods. The nonspecific staining control used normal mouse IgG. C5/D5 staining of colonic crypts and lamina propria leukocytes was performed. The colonic lumen ("L") was closest to the apical aspect of the intestinal epithelium ("E"). The basal and lateral aspects of the epithelium were strongly labelled. There was a noticeable absence of labeling of the apical surface. Beneath the epithelium is the interstitium ("I") or lamina propria which showed abundant inflammatory cells staining positively with C5/D5 IgG.

In summary, there was minimal background staining with irrelevant IgG in T84 monolayers or frozen colonic tissue sections respectively. In contrast, the en face fluorescent staining pattern on T84 cell monolayers viewed in a subjunctional plane revealed a typical "chicken wire" or basolateral staining pattern. While the apical surface of T84 cells showed very little staining with C5/D5 IgG, permeabilization of monolayers after fixation resulted in significant intracellular staining. To confirm the relevance of the localization findings in T84 cells, similar studies were performed on 1μ frozen sections of normal human colon. Strong labeling of the basolateral aspect of normal human colonic epithelium ("E") was observed. Also in agreement with the staining results on T84 monolayers was a lack of staining on the apical surface of natural human colonic epithelium. In addition, the results demonstrated labeling of mononuclear cells in the lamina propria. The majority of the lamina propria cells staining with the antibody appeared to be leukocytes.

(5) Relative Contributions of Neutrophil and Epithelial C5/D5 Antigen (CD47) to Transepithelial Migration

Since it is clear that the antigen defined by mAb C5/D5 is expressed by both epithelia and PMN, experiments were performed to determine the relative contributions of epithelial versus PMN CD47 on transepithelial migration. To exclude the possibility that C5/D5 IgG influenced transmigration by crosslinking epithelial cells to PMN, experiments using Fab' fragments of C5/D5 IgG were performed (Figure 4). PMN transepithelial migration in the absence of antibody was no different than migration in the presence of a control binding antibody W6/32 or

the same concentration of $F(ab')_2$ and Fab' prepared from normal mouse IgG (27±4.2 vs 27.3±4.1. 25.2±1.2 and 23.8±4x10^4 migrated PMN for no antibody vs W6/32, CTL $F(ab')_2$ and Fab' respectively, NS). In contrast, transmigration in the presence of C5/D5 $F(ab')_2$ and Fab' was inhibited by 93 and 85% respectively (25.2±1.2 vs 1.7±0.7x10^4 and 23.8±4 vs 3.6±0.5x10^4 migrated PMN for CTL vs C5/D5 $F(ab')_2$ and CTL vs C5/D5 Fab' respectively; $p<.01$). Such results effectively rule out inhibition caused by antibody mediated PMN - epithelial crosslinking or antibody mediated interactions with PMN Fc receptors.

Having excluded Fc and/or crosslinking mediated interactions as possible mechanisms of the observed inhibitory effects of C5/D5 on PMN transepithelial migration, experiments were performed to determine the relative contribution(s) of epithelial versus PMN CD47 to the transepithelial migration response. In such experiments, PMN or T84 monolayers were first pre-incubated with control or C5/D5 IgG and then extensively washed in HBSS to remove unbound mAb. Washed, antibody pretreated cells were then used in standard transmigration assays. As shown in Figure 5A, preincubation of inverted T84 monolayers with C5/D5 IgG resulted in 90 and 72% inhibition of transmigration when compared to antibody controls (20.4±1.7 vs 2±0.6 and 5.8±2.9x10^4 migrated PMN for CTL vs 50μg/ml and 25μg/ml C5/D5 IgG preincubation respectively; $p< .005$). However, as shown in Figure 5B, preincubation of PMN with C5/D5 IgG was also highly effective in inhibiting subsequent transepithelial migration (23.8±2.3 vs 0.08±2.17x10^4 migrated PMN for CTL vs C5/D5 IgG preincubation respectively; $p<.001$). Such results suggest that both PMN and epithelial associated CD47 may play roles in PMN transepithelial migration. The possibility that C5/D5 could directly affect PMN migration was confirmed in assays of the effects of C5/D5 on fMLP (10 nM gradient) induced PMN migration across acellular, collagen - coated permeable supports. As shown in Figure 5C, transmigration of PMN across filters in the absence of epithelia was also effectively inhibited by C5/D5 IgG (26.9±0.61 vs 1.28±0.1x10^4 migrated PMN for CTL vs migration in the presence of C5/D5 IgG respectively; $p<.001$).

Experiments were also performed both to examine the selectivity of C5/D5 - mediated inhibition of neutrophil function and to exclude the possibility that C5/D5 - mediated inhibition of PMN migration was a consequence of C5/D5 - mediated activation (i.e. due to desensitization). As shown in Table 1 (below), treatment of PMN with saturating concentrations of C5/D5 IgG did not influence PMN superoxide production or degranulation. In the presence of antibody, addition of saturating concentrations of fMLP resulted in superoxide production which

was indistinguishable from that in the absence of antibody. Furthermore, addition of C5/D5 IgG failed to induce the release of primary or secondary granules, nor did it augment or inhibit degranulation after stimulation with fMLP.

EXAMPLE 1 Table 1 - Effect of C5/D5 IgG on Neutrophil O_2-Production and Degranulation

Condition	Oxidase activity (nmol O_2-/10^6 PMN)	2°Granules; lactoferrin	1°Granules; myeloperoxidase
C5/D5 IgG	0	0.12	0.02
C5/D5 + fMLP	8.8	0.96	0.03
fMLP	9.3	0.97	0.03
PMA	15.8	1.49	0.03
W6/32	0	0.13	0.03
W6/32 + fMLP	9.0	0.73	0.03
PMN only	0	0.12	0.03
dHCB + fMLP			.074
Solubilized PMN			1.49

In regard to Table 1, suspensions of 10^6/ml PMN were preincubated for 10 min with 10 μg/ml C5/D5 IgG or control W6/32 IgG before stimulation for 5 min with fMLP (100 nM). Controls included stimulation with fMLP alone (5 min), PMA (phorbol myristate acetate) alone (5 min; 100 ng/ml) or preincubation with dihydrocytochalasin B (dHCB) (4 min at 5 mg/ml) followed by fMLP (5 min). For O_2-assays, PMN were suspended in cytocrome C buffer in the presence or absence of superoxide dismutase and catalase, and stimulated for 5 min. The superoxide dismutase inhibitable reduction of cytochrome C was then determined on the cell-free supernatants as described above. Lactoferrin was determined by ELISA, and myeloperoxidase by enzymatic activity as described above. The values reported above represent optical density following substrate addition. Each value was determined from 1 ml of cells (10^6 PMN).

(6) Effects of C5/D5 IgG on transendothelial migration.

We tested C5/D5 for effects on PMN migration across monolayers of HUVECS. As shown in Figure 6, migration of PMN across monolayers of HUVECS was markedly inhibited (91%) by C5/D5 IgG (3.8±2.7 vs 45.8±4.4x10^4 migrated PMN for C5/D5 vs W6/32 ctl respectively; $p<.001$).

C. Summary of Results

Little is known about the molecular interactions between PMN and columnar epithelia during transepithelial migration. The experiments described above were performed to obtain probes useful in further characterizing this important process. In this study we have raised and characterized a monoclonal antibody, C5/D5, which largely abolishes the ability of PMN to cross monolayers of the polarized crypt-like human intestinal epithelial cell line, T84. Although CD11b/CD18-mediated PMN adhesion to the epithelial surface appears to mediate initial contacts between PMN and columnar epithelial cells (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479) and is required for consummation of the transmigration response (Parkos, C.A. et al. (1991) J. Clin. Invest. 88:1605-12), the C5/D5 antigen does not appear to represent a ligand for CD11b/CD18. The C5/D5 antigen is shown to represent CD47, an unusual member of the immunoglobulin superfamily. In keeping with the broad tissue distribution of CD47, previously reported to be expressed on leukocytes, platelets, endothelial cells, placenta, ovarian cancer cells and variably on epithelia (Brown, E.L. et al. (1990) J Cell Biol. 111:2785-94; Campbell, I.G. et al. (1992) Cancer Res. 52:5416-20; Favaloro, E.J. (1993) Immunol Cell Biol. 71:571-81; Mawby, W.J. et al. (1994) Biochem J. 304:525-30), we now report CD47 expression on the basolateral surface of human colonic epithelial cells (cultured lines and natural tissue) and

provide evidence that implicates contributions of both PMN and epithelial - derived CD47 in the process of transepithelial migration.

The results described herein suggest that CD47 influences neutrophil-endothelial interactions. In particular, Example 1 confirms the importance of CD47 in transendothelial migration as demonstrated by the marked inhibitory effect of C5/D5 IgG on PMN migration across monolayers of HUVECS in response to fMLP. In addition, the Examples demonstrate that the inhibitory effects observed with C5/D5 are not mediated by Fc interactions or cell-cell cross-linking due to shared epitopes.

Given the columnar height of intestinal epithelial cells, migration of PMN across the paracellular space appears to be a complex process. Since C5/D5 IgG failed to inhibit PMN-epithelial adhesion or T84 cell adhesion to CD11b/CD18, it appears that the CD47 - mediated event(s) occur distal to at least one CD11b/CD18 - dependent adhesive interaction. Such results are schematized in Figure 7. In this multistep model of transepithelial migration, PMN, after extravasation, initially adhere to the basolateral aspect of intestinal epithelial cells in a CD11b/CD18 - mediated fashion. Such events are readily captured in modified epithelial - PMN adhesion assays (Parkos, C.A. et al. (1995) Am J. Physiol. 268:C472-C479) or in assays of epithelial adhesion to purified CD11b/CD18. Subsequently, PMN migrate over the extended lateral surface of intestinal epithelial cells in a manner which is dependent, at least in part, on CD47. Transmigrating PMN then disrupt the tight junction (Nash, S. et al. (1987) J. Clin. Invest. 80:1104-13; Nash, S. et al. (1988) Lab. Invest. 59:531-7) and enter the lumen of the crypt, forming a crypt abcess. Such a model would predict that inhibition of CD47-mediated transmigration would result in an accumulation of PMN within the epithelium. Indeed, we have observed increased monolayer - associated PMN in our transwell assays using C5/D5 IgG. These results have been accompanied by dramatic inhibition of the flux of PMN into the lower reservoir of the transwell device.

EXAMPLE 2 - Identification of Epitopic Peptides

The epitope on CD47 which reacts with the C5/D5 antibody is determined using phage display techniques as described in Burritt, J.B. et al. (1995) J. Biol. Chem. 270:16974-80 and Smith, G.P. et al. (1993) Meth. Enzymol. 217:228-257. Various libraries can be used for this purpose. For example, the phage library described in the above-noted Burritt reference consists of a genetically engineered filamentous bacteriophage into which nonapeptides with random amino acid sequences have been inserted. To determine the peptide epitope for a specific

antibody such as C5/D5, the antibody is immobilized (typically on sepharose beads) followed by incubation with the phage library. Phage which have sequences similar to the C5/D5 epitope bind to the antibody and are eluted, expanded and sequenced. From the nonapeptide sequences obtained for the binding phage, precise amino acid sequence information regarding the nature of the antibody epitope is determined. Once sequence information is obtained, the relevance of such sequence is tested in adhesion and, optionally, transmigration inhibition assays. Such assays involve demonstrating that the sequence of interest inhibits antibody binding to its antigen. Typically the peptides of interest are synthesized or grown up in large quantities of phage using recombinant methods.

It should be understood that the preceding is merely a detailed description of certain preferred embodiments. It therefore should be apparent to those skilled in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention.

All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

A Sequence Listing is presented below and is followed by what is claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: BRIGHAM AND WOMEN'S HOSPITAL, INC.

(B) STREET: 75 FRANCIS STREET

(C) CITY: BOSTON

(D) STATE: MASSACHUSETTS

(E) COUNTRY: UNITED STATES OF AMERICA

(F) POSTAL CODE: 02115

(ii) TITLE OF INVENTION: ANTIBODIES FOR MODULATING CD47-MEDIATED NEUTROPHIL TRANSMIGRATION

(iii) NUMBER OF SEQUENCES: 35

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: WOLF, GREENFIELD & SACKS, P.C.

(B) STREET: 600 ATLANTIC AVENUE

(C) CITY: BOSTON

(D) STATE: MASSACHUSETTS

(E) COUNTRY: UNITED STATES OF AMERICA

(F) POSTAL CODE: 02210

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: HEREWITH

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/593,815

(B) FILING DATE: 30-JAN-1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Plumer, Elizabeth R.

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(C) REFERENCE/DOCKET NUMBER: B0801/7047WO

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-720-3500

(B) TELEFAX: 617-720-2441

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 142 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Trp Pro Leu Val Ala Ala Leu Leu Leu Gly Ser Ala Cys Cys Gly
1 5 10 15

Ser Ala Gln Leu Leu Phe Asn Lys Thr Lys Ser Val Glu Phe Thr Phe
20 25 30

Cys Asn Asp Thr Val Val Ile Pro Cys Phe Val Thr Asn Met Glu Ala
35 40 45

Gln Asn Thr Thr Glu Val Tyr Val Lys Trp Lys Phe Lys Gly Arg Asp
50 55 60

Ile Tyr Thr Phe Asp Gly Ala Leu Asn Lys Ser Thr Val Pro Thr Asp
65 70 75 80

Phe Ser Ser Ala Lys Ile Glu Val Ser Gln Leu Leu Lys Gly Asp Ala
85 90 95

Ser Leu Lys Met Asp Lys Ser Asp Ala Val Ser His Thr Gly Asn Tyr
100 105 110

Thr Cys Glu Val Thr Glu Leu Thr Arg Glu Gly Glu Thr Ile Ile Glu
115 120 125

Leu Lys Tyr Arg Val Val Ser Trp Phe Ser Pro Asn Glu Asn
130 135 140

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Phe Lys Gly Arg Asp Ile Tyr Thr Phe Asp Gly Ala Leu Asn Lys Ser
1 5 10 15

Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile Glu Val Ser Gln Leu
20 25 30

Leu Lys Gly Asp Ala Ser Leu Lys Met
35 40

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asn Lys Thr Lys Ser Val Glu Phe Thr Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asn Lys Thr Lys Ser Val Glu Phe Thr Phe Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Lys Thr Lys Ser Val Glu Phe Thr Phe Cys Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asn Lys Thr Lys Ser Val Glu Phe Thr Phe Cys Asn Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Phe Asn Lys Thr Lys Ser Val Glu Phe Thr Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Phe Asn Lys Thr Lys Ser Val Glu Phe Thr Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide.

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Leu Phe Asn Lys Thr Lys Ser Val Glu Phe Thr Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Ser Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile Glu Val Ser
1 5 10 15
Gln

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Ser Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile Glu Val Ser
1 5 10 15
Gln Leu

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear .

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Lys Ser Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile Glu Val Ser
1 5 10 15

Gln Leu Leu

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Lys Ser Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile Glu Val Ser
1 5 10 15

Gln Leu Leu Lys
20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Asn Lys Ser Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile Glu Val
1 5 10 15

Ser Gln

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Asn Lys Ser Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile Glu
1 5 10 15

Val Ser Gln

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Leu Asn Lys Ser Thr Val Pro Thr Asp Phe Ser Ser Ala Lys Ile
1 5 10 15

Glu Val Ser Gln
20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Ser Ala Lys Ile Glu
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Ser Ala Lys Ile Glu Val
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ser Ser Ala Lys Ile Glu Val Ser
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ser Ser Ala Lys Ile Glu Val Ser Gln
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Phe Ser Ser Ala Lys Ile Glu
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Asp Phe Ser Ser Ala Lys Ile Glu
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Thr Asp Phe Ser Ser Ala Lys Ile Glu
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Lys Gly Asp Ala Ser Leu Lys Met Asp Lys Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Lys Gly Asp Ala Ser Leu Lys Met Asp Lys Ser Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Lys Gly Asp Ala Ser Leu Lys Met Asp Lys Ser Asp Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Lys Gly Asp Ala Ser Leu Lys Met Asp Lys Ser Asp Ala Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide.

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Leu Lys Gly Asp Ala Ser Leu Lys Met Asp Lys Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Leu Leu Lys Gly Asp Ala Ser Leu Lys Met Asp Lys Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Gln Leu Leu Lys Gly Asp Ala Ser Leu Lys Met Asp Lys Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Phe Ser Ser Ala Lys Ile Glu Val Ser Gln Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Phe Ser Ser Ala Lys Ile Glu Val Ser Gln Leu Leu Lys Gly

1 5 10

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Asp Phe Ser Ser Ala Lys Ile Glu Val Ser Gln Leu Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Ile Glu Val Ser Gln Leu Leu Lys
1 5

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Ser Thr Val Pro Thr Asp Phe Ser Ser Ala
1 5 10

Claims

1. A composition comprising an inhibitory agent selected from the group consisting of a monoclonal antibody having ATCC Accession No. HB-12021, a functionally active fragment of the antibody having ATCC Accession No. HB-12021, and a monoclonal antibody having the characteristics of the antibody having ATCC Accession No. HB-12021.

2. The composition of claim 1, wherein the composition comprises the monoclonal antibody having ATCC Accession No. HB-12021.

3. The composition of claim 1, wherein the composition comprises the functionally active fragment of the antibody having ATCC Accession No. HB-12021, wherein the fragment is selected from the group consisting of an $F(ab')_2$ fragment, an Fab fragment, Fv fragment and an Fd fragment of the antibody having ATCC Accession No. HB-12021.

4. The composition of claim 1, further comprising a pharmaceutically acceptable carrier, wherein the inhibitory agent is present in a therapeutically effective amount.

5. The composition of claim 1, wherein the composition comprises the monoclonal antibody having the characteristics of the monoclonal antibody having ATCC Accession No. HB-12021.

6. The composition of claim 5, wherein the characteristics of the antibody comprise an inhibitory concentration in a neutrophil transmigration assay that results in at least 65% inhibition of neutrophil transmigration in the neutrophil transmigration assay and wherein the inhibitory concentration is between 0.1 ug/ml and 50 ug/ml, inclusive.

7. The antibody of claim 6, wherein the transmigration assay measures the transmigration of neutrophils across a support selected from the group consisting of a cell layer, an extracellular matrix and a filter.

8. The antibody of claim 7, wherein the transmigration assay measures the transmigration of neutrophils across a cell layer.

9. The antibody of claim 8, wherein the transmigration assay measures transmigration across a polarized cell layer.

10. The antibody of claim 8, wherein the antibody inhibits transmigration across the cell layer in a bidirectional fashion.

11. The antibody of claim 10, wherein the antibody does not inhibit CD11b/CD18-mediated adhesion of the neutrophil to the cells of the cell layer.

12. The antibody of claim 5, wherein the characteristics of the antibody comprise specificity for an epitope that is specifically recognized by the monoclonal antibody having ATCC Accession No. HB-12021.

13. The antibody of claim 12, wherein the epitope is defined by an amino acid sequence containing between three and twenty amino acids of SEQ. I.D. No. 1.

14. The antibody of claim 13, wherein the epitope contains the sequence SSAKIE.

15. The antibody of claim 13, wherein the amino acid sequence is selected from the group consisting of SEQ. I.D. Nos. 2-34 and 35.

16. The antibody of claim 5, wherein the characteristics of the antibody comprise an antigen binding site having an amino acid sequence that is identical to the amino acid sequence of the antigen binding site of the monoclonal antibody having ATCC Accession No. HB-12021.

17. An isolated peptide selected from the group consisting of SEQ. I.D. Nos. 1-34 and 35.

18. A method for inhibiting the migration of a CD47-expressing cell across a support selected from the group consisting of a cell layer, an extracellular matrix and a filter, the method comprising contacting at least one of the CD47-expressing cells and the support with an inhibitory agent prior to transmigration, wherein the inhibitory agent is selected from the group consisting of a monoclonal antibody having ATCC Accession No. HB-12021, a functionally active fragment of the antibody having ATCC Accession No. HB-12021, and a monoclonal antibody having the characteristics of the antibody having ATCC Accession No. HB-12021.

19. A method for modulating an immune response in a subject comprising:

administering to the subject a pharmaceutical composition containing a pharmaceutically acceptable carrier and an inhibitory agent that inhibits the transmigration of a neutrophil across a cell layer or extracellular matrix, wherein the inhibitory agent is selected from the group consisting of a monoclonal antibody having ATCC Accession No. HB-12021, a functionally active fragment of the antibody having ATCC Accession No. HB-12021, and a monoclonal having the characteristics of the antibody having ATCC Accession No. HB-12021, wherein the inhibitory agent is present in a therapeutically effective amount to modulate the immune response.

20. The method of claim 19, further comprising coadministering an "adhesion inhibitory agent" to the subject.

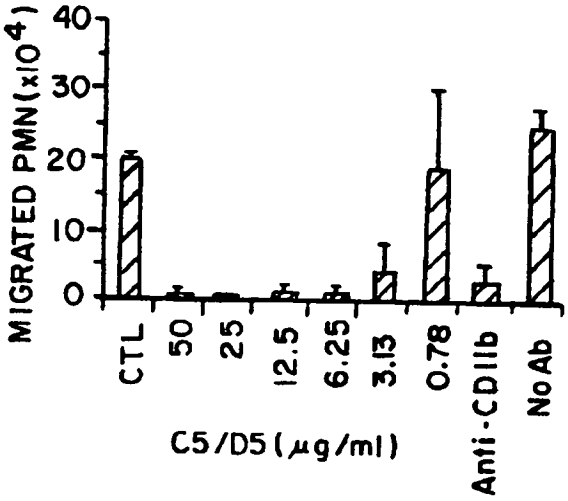


FIG. 1A

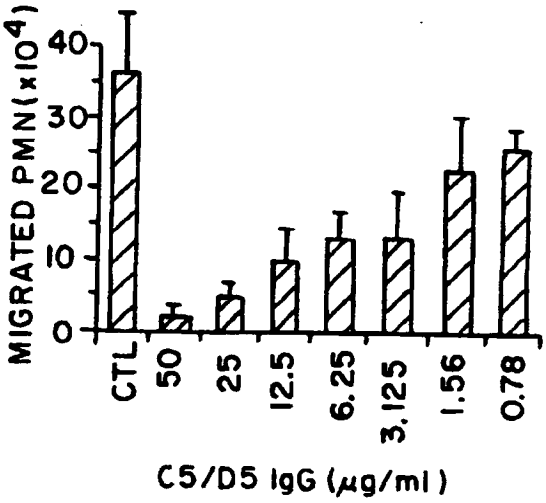


FIG. 1B

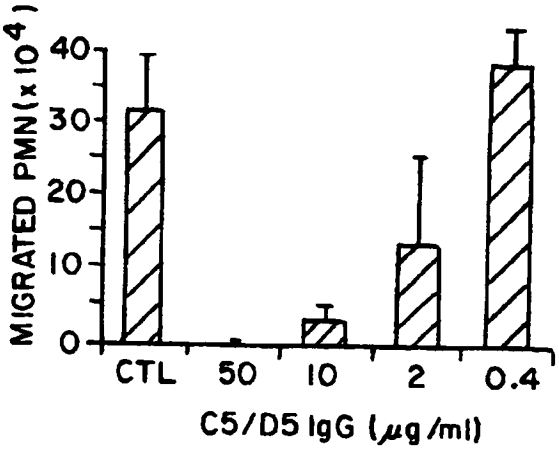


FIG. 1C

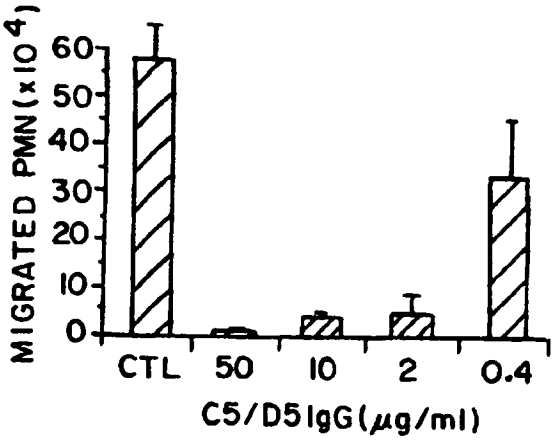


FIG. 1D

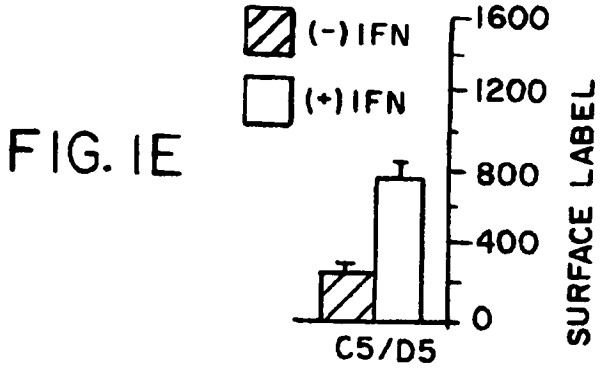
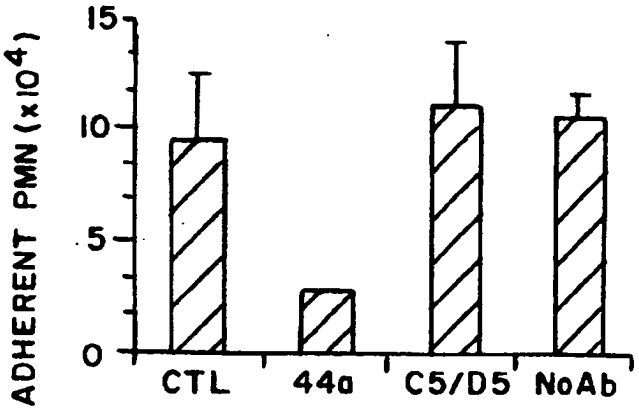


FIG. 1E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15
10
5
0
CTL
44a
C5/D5
NoAb

FIG. 2A

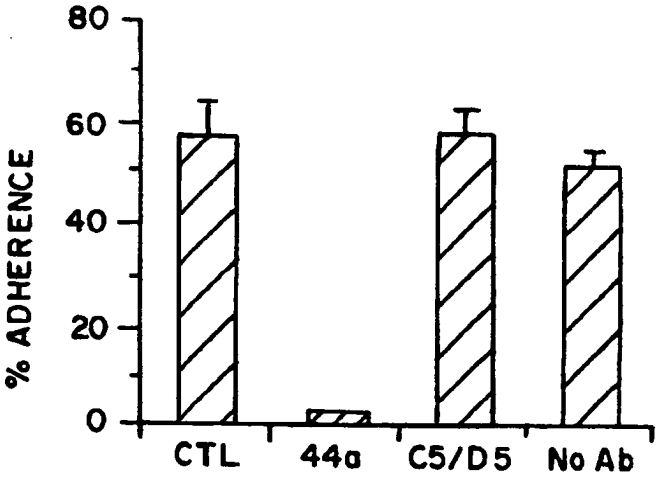
% ADHERENCE
80
60
40
20
0
CTL
44a
C5/D5
No Ab

FIG. 2B

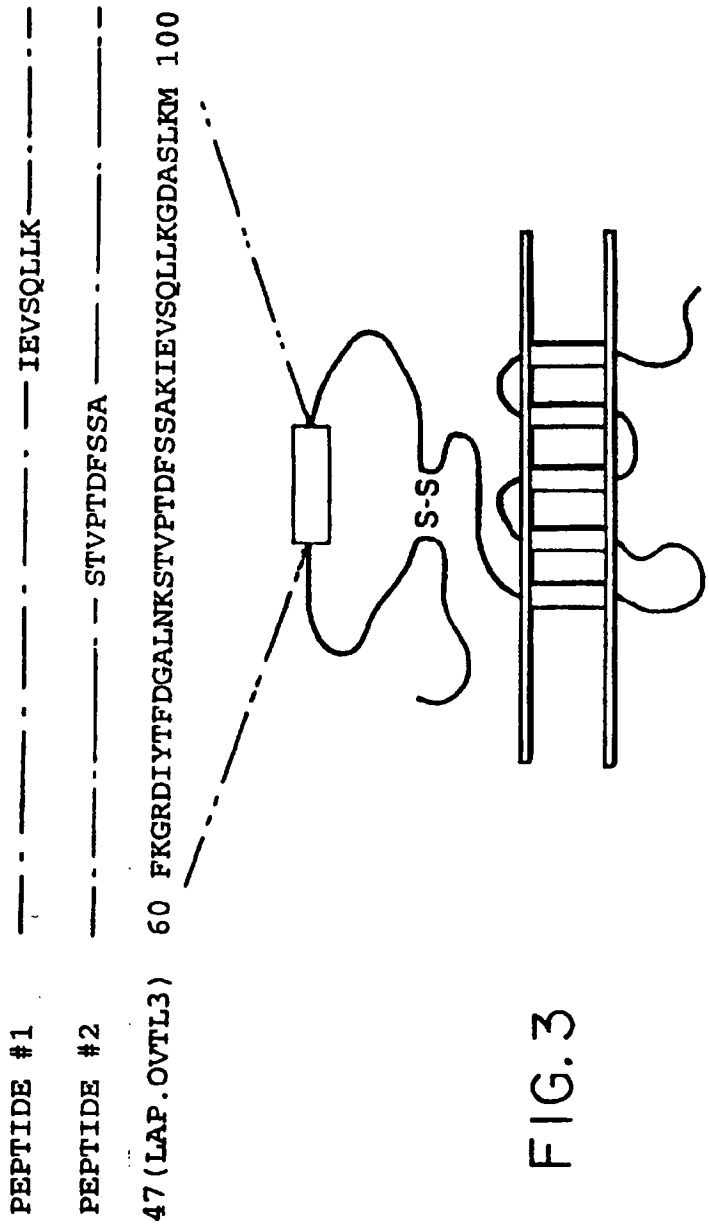
PEPTIDE #1
IEVSQLLK
PEPTIDE #2
STVPTDFSSA
47(LAP.OVTL3)
60 FKGRDIYTFDGALNKSTVPTDFSSAKIEVSQLLKGDASLKM 100
S-S

FIG. 3

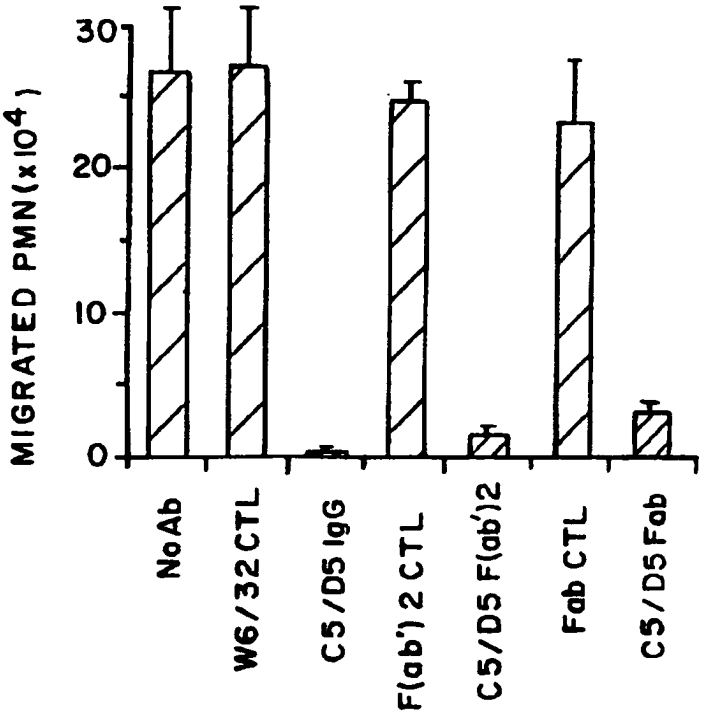
MIGRATED PMN(x10⁴)
30
20
10
0
NoAb
W6/32 CTL
C5/D5 IgG
F(ab')2 CTL
C5/D5 F(ab')2
Fab CTL
C5/D5 Fab

FIG. 4

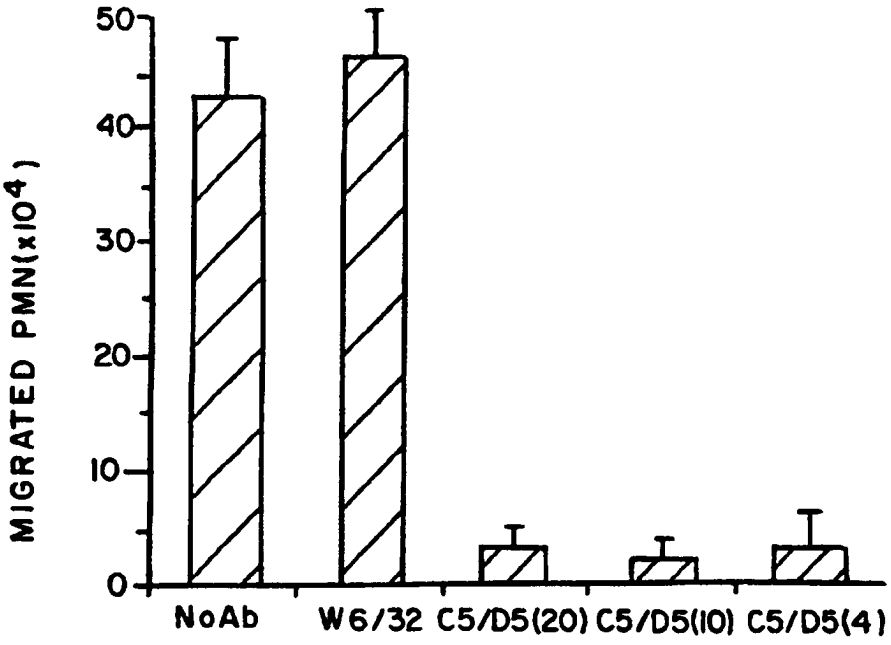
MIGRATED PMN(x10⁴)
50
40
30
20
10
0
NoAb
W6/32
C5/D5(20)
C5/D5(10)
C5/D5(4)

FIG. 6

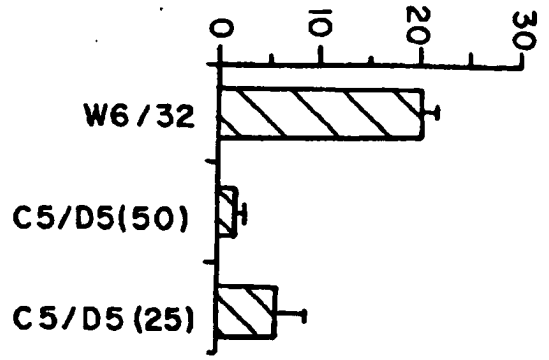

FIG.5A

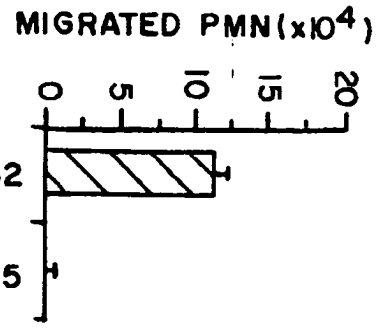

FIG.5B

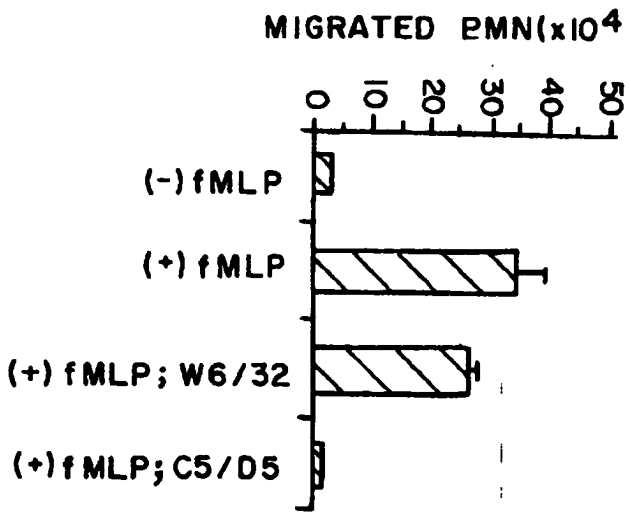

FIG.5C

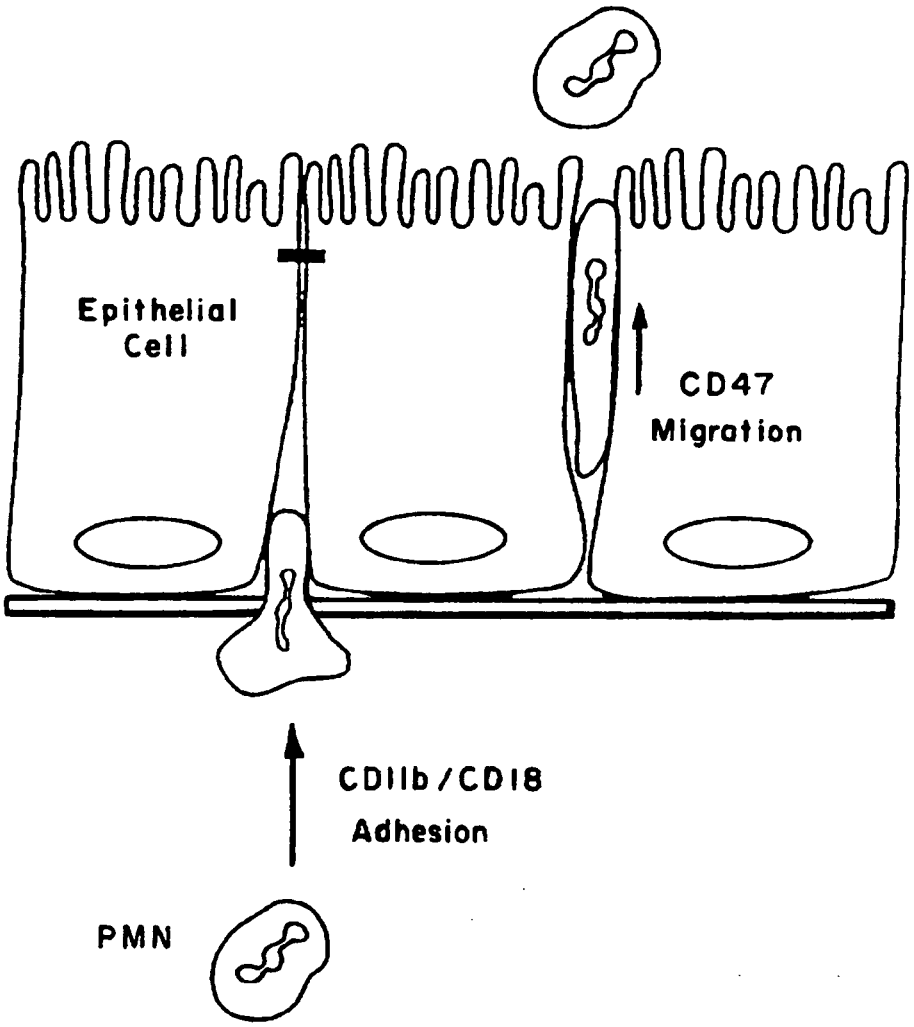
LUMEN
Epithelial Cell
CD47 Migration
CD11b / CD18 Adhesion
PMN

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01340

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/395; G01N 33/558; C07K 1/00
US CL :424/141.1; 436/514; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/141.1; 436/514; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE, EMBASE, CAPLUS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,057,604 A (BROWN) 15 October 1991, see entire document.	1-20
Y	GRESHAM et al. A novel member of the integrin receptor family mediates Arg-Gly-Asp-stimulated neutrophil phagocytosis. Journal of Cell Biol. May 1989. Vol. 108, pages 1935-43, see entire document.	1-20
Y	COOPER et al. Transendothelial migration of neutrophils involves integrin-associated protein (CD47). April 1995. Proc. Natl. Acad. Sci. USA, Vol. 92, pages 3978-92, see entire document.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 FEBRUARY 1997	Date of mailing of the international search report 17 MAR 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RAY F. EBERT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01340

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PARKOS et al. Neutrophil migration across a cultured intestinal epithelium. Journal of Clin. Invest. November 1991. Vol. 88, pages 1605-12, see entire document.	1-20
Y	GODING, J.W. Monoclonal antibodies: Principles and Practice. December 1986. New York: Academic Press, pages 125-133, see entire document.	3